UNIVERSITY OF ALBERTA

CHARACTERISTICS OF IMMUNE PARAMETERS BEFORE AND AFTER A SINGLE MANTOUX TEST

BY

VALERIE NORONHA MENEZES KREUTZ

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A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of the
requirements for the degree of Master of Science.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characteristics of Immune Parameters Before and After a Single Mantoux Test submitted by Valerie Noronha Menezes Kreutz in partial fulfillment of the requirements for the degree of Master of Science

H. Pabst

Dr. Henry Pabst

Dr. Tim Mosmann

Dr. John Elliott

Dr. Gary Lopaschuk

April 6/98

DEDICATION

To my beloved husband, Fernando, and my loving sons, Rodrigo and Diogo.

ABSTRACT

This study analyzed the effect of Mantoux testing upon some immune parameters.

Blood was taken from fifteen medical personnel before and 48 hours after a Mantoux test. Peripheral blood mononuclear cells (PBMC) were stimulated with purified protein derivative (PPD), phytohemagglutinin (PHA), and also candida and tetanus antigens. Supernatants were harvested at days 1, 2, 4, and 6 of culture and cytokine content was measured by ELISAs. Thymidine incorporation (TI) was measured at day six . Percentages of CD4, CD8 and CD14 positive cells in the PBMC samples were measured by FACScan.

There was IFNγ production upon PPD stimulation in all subjects tested. There was no significant difference in the IFNγ production and TI responses to PPD after Mantoux testing and the responses were consistent for a given subject. There was a strong correlation between the IFNγ and TI responses upon stimulation with PPD. A larger subject sample is needed to identify any distinct pattern between groups with different Mantoux status.

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TABLE OF CONTENTS

1. INTRODUCTION 1

1.1 OVERVIEW OF TUBERCULOSIS 1

1.2. PPD 5

1.2.1. HISTORY 5

1.2.2. PRODUCTION 5

1.2.3. OTHER MYCOBACTERIAL PREPARATIONS 6

1.3. MANTOUX TEST 7

1.3.1. READING 8

1.3.2. CORRELATION WITH PROTECTION 9

1.3.3. STUDIES DONE IN THE SKIN 19

1.4. CYTOKINES AND PROTECTION 20

1.4.1. THE TH1/TH2 MODEL 20

1.4.2. CYTOKINES AND OTHER IMMUNE PARAMETERS DURING TUBERCULOSIS 21

1.4.3. RELATIONSHIP BETWEEN MANTOUX STATUS AND CYTOKINES AND OTHER IMMUNE PARAMETERS 25

1.5. OBJECTIVES 27

2. MATERIALS AND METHODS 27

2.1. ISOLATION OF LYMPHOCYTES 28

2.2. TISSUE CULTURE ASSAYS 29

2.2.1. PROLIFERATION ASSAYS *29*

2.2.2. CYTOKINE PRODUCTION *30*

2.2.3. ANTIGENS *30*

2.3. ELISAS 31

2.3.1. REAGENTS *32*

2.3.2. GENERAL ELISA PROCEDURE *32*

2.3.3. ASSAY OPTIMIZATION *34*

2.3.4. FINAL FORMAT *39*

2.4. IMMUNOPHENOTYPING 57

3. RESULTS 59

3.1. MANTOUX RESULTS 59

3.2. PPD RESPONSE 59

3.2.1 IFNγ PRODUCTION *59*

3.2.2. THYMIDINE INCORPORATION (TI) *60*

3.2.3. CORRELATIONS BETWEEN TI AND IFNγ RESPONSE *61*

3.2.4. OTHER CYTOKINES *61*

3.3. CANDIDA AND TETANUS RESPONSE 62

3.3.1. IFNγ PRODUCTION *62*

3.3.2. THYMIDINE INCORPORATION *62*

3.3.3. OTHER CYTOKINES *62*

3.4. PHA RESPONSE 63

3.4.1 IFNγ RESPONSE *63*

3.4.2 THYMIDINE INCORPORATION *63*

3.4.3 OTHER CYTOKINES *63*

3.5. CONTROL CULTURES 63

3.6. IMMUNOPHENOTYPING RESULTS 63

4. DISCUSSION AND CONCLUSIONS 91

4.1. DISCUSSION 91

4.2. CONCLUSIONS 101

5. REFERENCES 103

6. APPENDIX 125

LIST OF FIGURES

FIGURE PAGE

FIGURE 3-1. MANTOUX RESULTS.....65

FIGURE 3-2. IFNγ PRODUCTION WITHOUT ANTIGENIC STIMULATION (CONTROL CULTURES).....66

FIGURE 3-3. IFNγ PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PPD.....67

FIGURE 3-4. IFNγ PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD.....68

FIGURE 3-5. IFNγ PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD.....69

FIGURE 3-6. IFNγ PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PDD BEFORE AND AFTER MANTOUX.....70

FIGURE 3-7. IFNγ PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD BEFORE AND AFTER MANTOUX.....71

FIGURE 3-8. IFNγ PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD BEFORE AND AFTER MANTOUX.....72

FIGURE 3-9. IFNγ PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD BEFORE AND AFTER MANTOUX. SIZE OF SYMBOLS CORRESPOND TO SIZE OF MANTOUX REACTION.....73

FIGURE 3-10. IFNγ PRODUCTION WITHOUT ANTIGENIC STIMULATION (CONTROL CULTURES). SIZE OF SYMBOLS CORRESPOND TO SIZE OF MANTOUX REACTION.....74

FIGURE 3-11. TI WITHOUT ANTIGENIC STIMULATION (CONTROL CULTURES).....75

FIGURE 3-12. TI UPON STIMULATION WITH THREE DIFFERENT CONCENTRATIONS OF PPD.....76

FIGURE 3-13. TI UPON STIMULATION WITH THREE DIFFERENT DOSES OF PPD.....77

FIGURE 3-14. IFNγ PRODUCTION VERSUS TI UPON STIMULATION WITH 2 μG/ML OF PPD.....78

FIGURE 3-15. IFNγ PRODUCTION VERSUS TI UPON STIMULATION WITH 10 μG/ML OF PPD.....79

FIGURE 3-16. IFNγ PRODUCTION VERSUS TI BEFORE AND AFTER MANTOUX TEST IN DAY SIX SUPERNATANTS FROM CULTURES STIMULATED WITH PPD 10 μG/ML.....80

FIGURE 3-17. IFNγ PRODUCTION VERSUS TI UPON STIMULATION WITH 50 μG/ML OF PPD.....81

FIGURE 3-18. IL5 PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD.....82

FIGURE 3-19. IL13 PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD.....83

FIGURE 3-20. TNFα PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD.....84

FIGURE 3-21. IFNγ PRODUCTION UPON STIMULATION WITH TETANUS.....85

FIGURE 3-22. IFNγ PRODUCTION UPON STIMULATION WITH CANDIDA.....86

FIGURE 3-23. IFNγ PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PHA.....87

FIGURE 3-24. PERCENTAGE OF $CD4^+$ AND $CD8^+$ T CELLS IN THE PBMC SAMPLES BEFORE AND AFTER MANTOUX.....88

FIGURE 3-25. PERCENTAGE OF $CD14^+$ CELLS IN THE PBMC SAMPLES BEFORE AND AFTER MANTOUX.....89

FIGURE 3-26. IFNγ PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD BEFORE AND AFTER MANTOUX WITH STANDARD DEVIATIONS.....90

FIGURE A-1. IL4 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD.....126

FIGURE A-2. IL4 PRODUCTION UPON STIMULATION WITH 50 μG/ML OF PPD.....127

FIGURE A-3. IL5 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD.....128

FIGURE A-4. IL5 PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PPD.....129

FIGURE A-5. IL13 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD.....130
FIGURE A-6. TNFα PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PPD.....131
FIGURE A-7. TNFα PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PPD.....132
FIGURE A-8. TI UPON STIMULATION WITH CANDIDA AND TETANUS.....133
FIGURE A-9. IL4 PRODUCTION UPON STIMULATION WITH CANDIDA.....134
FIGURE A-10. IL4 PRODUCTION UPON STIMULATION WITH TETANUS.....135
FIGURE A-11. IL5 PRODUCTION UPON STIMULATION WITH CANDIDA.....136
FIGURE A-12. IL5 PRODUCTION UPON STIMULATION WITH TETANUS.....137
FIGURE A-13. IL13 PRODUCTION UPON STIMULATION WITH CANDIDA.....138
FIGURE A-14. IL13 PRODUCTION UPON STIMULATION WITH TETANUS.....139
FIGURE A-15. TNFα PRODUCTION UPON STIMULATION WITH CANDIDA.....140

FIGURE A-16. TNFα PRODUCTION UPON STIMULATION WITH TETANUS.....141
FIGURE A-17. IFNγ PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PHA.....142
FIGURE A-18. IFNγ PRODUCTION UPON STIMULATION WITH CANDIDA.....143
FIGURE A-19. TI UPON STIMULATION WITH TWO DIFFERENT DOSES OF PHA.....144
FIGURE A-20. TNFα PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PHA.....145
FIGURE A-21. TNFα PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PHA.....146
FIGURE A-22. IL5 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PHA.....147
FIGURE A-23. IL5 PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PHA.....148
FIGURE A-24. IL5 PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PHA.....149
FIGURE A-25. IL13 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PHA.....150
FIGURE A-26. IL4 PRODUCTION UPON STIMULATION WITH 2 μG/ML OF PHA.....151

FIGURE A-27. IL4 PRODUCTION UPON STIMULATION WITH 10 μG/ML OF PHA.....152
FIGURE A-28. IL5 PRODUCTION WITHOUT ANTIGENIC STIMULATION...153
FIGURE A-29. IL13 PRODUCTION WITHOUT ANTIGENIC STIMULATION..154
FIGURE A-30. TNFα PRODUCTION WITHOUT ANTIGENIC STIMULATION.....155

LIST OF ABBREVIATIONS

Ab	Antibody
ABTS	2.2'-azino-di[2-ethyl-benzthiazoline sulfonate]
AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guerin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CMI	Cellular mediated immunity
°C	Degrees centigrade
DTH	Delayed type hypersensitivity
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IFNγ	Interferon gamma
IL4	Interleukin 4
IL5	Interleukin 5
IL10	Interleukin 10
IL13	Interleukin 13
LP	Lymphoproliferative proliferation
mL	Milliliters
mm	Millimeters
Mtb	*Mycobacterium tuberculosis*
OD	Optical density
OT	Old tuberculin
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate-buffered saline
PE	Phycoerythrin
PHA-P	Phytohemagglutinin
PPD	Purified protein derivative
TB	Tuberculosis
TH1	T helper 1
TH2	T helper 2
TI	Thymidine incorporation
TMB	Tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TU	Tuberculin unit

1. INTRODUCTION

1.1 OVERVIEW OF TUBERCULOSIS

Tuberculosis (TB), long regarded as a declining disease, is back. It is assumed that about one-third of the world population is infected with *Mycobacterium tuberculosis* (Flesch, 1993). The case rates have risen dramatically in regions where human immunodeficiency virus infection is widespread. Outbreaks of multidrug-resistant disease underscore the need for novel approaches to treat and prevent tuberculosis. Global incidence and tuberculosis deaths are predicted to have an increase of 36% and 39% respectively by the year 2000 (Dolin,1994). Although BCG (Bacille Calmette-Guerin) vaccine was introduced in 1921 and now its use is widespread, its protection against TB is still a matter of controversy. Therefore, a more detailed understanding of the immune response to *M. tuberculosis* (M.tb), including definition of the T-cell subpopulations and cytokines that mediate resistance to tuberculous infection, would bring new insight into the development of strategies against TB.

Infection with *M. tuberculosis* is most commonly acquired by inhaling aerosolized bacteria. The majority of patients successfully contain the primary infection within 2-10 weeks and go on to develop a vigorous delayed type hypersensitivity (DTH) response. About 5-10% will develop progressive primary

disease in the 2 years immediately following infection and nearly all cases occur within 5 years of infection. If untreated, 50% of sputum positive patients will die within 5 years of diagnosis, 30% will be self-cured and the remaining 20% remain alive (Smith, 1995). The individuals who do not develop disease following infection apparently mount an effective immune response which can destroy the bacilli or limit their proliferation. The latter leads to a state of dormant TB that later in life can be reactivated (endogenous reinfection).

Does natural infection with M.tb confer lifelong immunity? The answer has been a subject of considerable controversy over the years (Ellner, 1997]. The difficulty of the diagnosis added to the state of dormancy that can be assumed by the disease make it difficult to define if exogenous reinfection really occurs. If exogenous reinfection does not exist, later periods of activity of tuberculosis appearing even years after a documented episode of the infection should be ascribed to endogenous reactivation. This assumption implies that M.tb infection may confer lifelong immunity to those who effectively get rid off the bacilli. Epidemiological studies done in the past suggested that both reinfection or reactivation of dormant TB can occur, the former depending on the risk of disease in the geographic area (Stead, 1967). Until very recently, the possibility of distinction between the two routes of disease has not existed, and it has been necessary to employ epidemiological modeling techniques to estimate their relative contributions to the total disease load in a population. Based on an epidemiological model from studies in the Netherlands, Sutherland *et al* (Sutherland, 1982) suggested that protection from a distant primary infection

occurs but is incomplete. The degree of protection conferred by a healed primary infection estimated from their epidemiological model was 63 % for males and 81 % for females. In the future, the development of molecular methods to characterize strains of M.tb, such as restricted fragment length polymorphism, will hopefully clarify such important issues.

Fundamental events in the pathogenesis of tuberculosis have been elucidated by the use of animal models of the disease. Much controversy surrounds the different approaches taken to establish an animal model of disease similar to human infection: the choice of animal, the route of infection, the dose of vaccination and challenge, the organism to use and the read-out system for protection, all influence the outcome and interpretation of the experiment. The animal model developed by Lurie (Rich,1951) appears important in elucidating the evolution of a primary lesion in the lungs after infection with M.tb. Rabbits and guinea pigs show cavitary lesions and caseous necrosis similar to human disease (Ehlers, 1994). Lurie's classic studies of tuberculosis in inbred strains of rabbits (Rich,1951) demonstrated the role of macrophages in the immune control of mycobacteria. In this model resistance to the establishment of tuberculosis is under genetic control. Seven days after the inhalation of human tubercle bacilli, the lungs of Lurie's resistant rabbits contained significantly fewer viable bacilli than did the lungs of susceptible rabbits (Dannenberg, 1991). Pathological studies from Lurie's animal models showed 4 stages in the development of pulmonary TB. In **stage 1** there is no bacillary growth. The bacillus is usually destroyed or inhibited by the mature

resident alveolar macrophage (AM) that ingests it and the infection is arrested. **Stage 2** occurs between 7 and 21 days after infection if the AM is incapable of controlling the inhaled bacilli. The bacilli grow logarithmically within the AM (or its progeny) until the latter is destroyed, releasing the bacilli. The bacillary load is then ingested by other AM and nonactivated macrophages from the bloodstream. In time, the latter will become solely responsible for the fate of the early primary tubercle in which the AMs rarely participate. The bacilli are at the center of the lesion while the AM are located peripherally. Even though macrophages in the lesions of resistant animals contain fewer bacilli and usually are located interstitially, the rate of multiplication of bacilli is the same in Lurie's resistant and susceptible rabbits. The same was observed by Smith (Smith, 1977) when comparing BCG-vaccinated and control guinea-pigs. Around 5 weeks after the initial infection **stage 3 occurs**. The logarithmic multiplication stops, the host becomes tuberculin positive and the tubercle has a caseous necrotic center. Now there are 20 to 30 times more bacilli in the lungs of susceptible than in resistant rabbits. In spite of this numerical difference, both resistant and susceptible hosts inhibit further bacillary growth just as efficiently. At this point Dannenberg (Dannenberg 1991, Dannenberg 1993, Dannenberg, 1994) separates the immune response into 2 types, tissue damage response (caseous necrosis, caused by DTH) and the macrophage-activating response (cellular mediated immunity--CMI). **Stage 4a** occurs in the susceptible rabbit, when there is liquefaction of the solid caseous focus. For the first time, the bacilli multiply profusely extracellularly. The material from the tubercle can be

discharged into the airways with bacillary dissemination to other parts of the lung or organs and into the environment. **Stage 4b** occurs in resistant rabbits: if the caseous center does not liquefy, the disease will be arrested, according to this interpretation, by CMI because further tissue destruction does not occur. This scenario occurs in healthy immunocompetent humans who show positive tuberculin reactions and yet no clinical (and often not even x-ray) evidence of the disease.

1.2. PPD

1.2.1. HISTORY

Old tuberculin (OT) was developed by Robert Koch and was a concentrated filtrate of broth in which tubercle bacilli had grown for 6 weeks (Rich, 1951). There were problems related to the crude nature of the product and that many components originating from the broth itself were concentrated with the mycobacterial proteins. Later, it became possible to use synthetic, protein-free media to support growth for tuberculin production. A much cleaner product, called purified protein derivative (PPD) was obtained by chemical fractionation of OT by Seibert and colleagues in 1932.

1.2.2. PRODUCTION

The production methods may vary from one producer to another, but essentially the products consist of proteins, released into the growth medium from stationary cultures of *M. tuberculosis*, that are recovered by precipitation with ammonium sulfate or trichloric acetic acid after heat inactivation, filtration, and concentration of the culture. PPD is standardized in terms of its biological reactivity as 'tuberculin units" (TU). The exact nature of the antigenic determinants in PPD has not been conclusively established although there is a report by Young and colleagues (Young, 1992) where they list some proteins isolated from such preparation that have been investigated by different research groups. By international agreement, the TU is defined as the activity contained in a specific weight of Seibert's PPD lot number 49608 in a specified buffer. This is PPD-S, the standard for tuberculin against which the potency of all products must be established by biological assay - i.e., by reaction size in humans. First strength tuberculin has 1 TU; intermediate strength has 5 TU ; and second strength has 250 TU. Bioequivalency of PPD products is not based on weight of the material but on comparative activity. In 1952, the Resent Institute (Copenhagen) had begun production of the master batch of PPD-RT (Resent Tuberculin) sponsored by the World Health Organization and United Nations Children's Fund. Two TU of PPD-RT23 are equivalent to five TU of PPD-S. It was agreed that UNICEF would make this preparation available for general use.

1.2.3. OTHER MYCOBACTERIAL PREPARATIONS

Many bacterial proteins are highly conserved, not only within the genus Mycobacterium but also in a broad range of other bacterial species. One example is the group of stress or heat shock proteins that are produced in abundant quantities by *M. tuberculosis* which exhibit at least 50% homology at the amino acid level with stress proteins from other bacterial species. Therefore, it is not surprising that PPD is not a fully species-specific reagent but is widely cross-reacting.

In an attempt to control this crossreactivity, due to the presence in many regions of environmental mycobacteria (Dascalopoulos, 1995), tuberculins from other mycobacteria were developed (Edwards, 1965) to try to distinguish between environmental mycobacterial exposure and *M. tuberculosis* infection. Mantoux studies using such preparations concomitantly fail to confirm such a distinction since both preparations (PPD and atypical mycobacteria) may generate overlapping sizes of induration (Baily, 1980).

1.3. MANTOUX TEST

The use of the tuberculin test as a diagnostic tool for TB was originally based on the Koch phenomenon. Robert Koch, in 1890, in his first description of tuberculin described the effect of the subcutaneous injection of this material into tuberculous humans and guinea pigs. Koch found that healthy human beings reacted scarcely or not at all to the subcutaneous injection of 0.1 mL of old

tuberculin but 0.01 mL injected subcutaneously into a tuberculous patient caused a severe general reaction associated with a rise in temperature starting 4 to 5 hours after injection and lasting for 12 to 15 hours. The injection site also became red and painful (reviewed in Turk,1980).

In 1910, Mantoux described the reaction to the intradermal (Mantoux test) as opposed to subcutaneous injection of tuberculin (Rich,1951). The reaction starts a few hours after injection as a white or rose colored infiltration. It increases in strength during the first 24 hours but does not attain its peak in the human until two days after injection. Mantoux described the reaction as a central nodule separated from a peripheral halo by an intermediary zone. The reaction at its height is red and indurated, sometimes with petechial hemorrhages at the center.

1.3.1. READING

The Mantoux test is done by the intradermal administration of 0.1 mL of PPD containing 5 TU into the flexor or dorsal surface of the forearm, about 4 inches below the antecubital area. Only induration is considered when interpreting the test. The diameter of induration is measured transversely to the long axis of the forearm and recorded in millimeters (mm). Persons with sensitivity to tuberculin are called "reactors"; however, not all reactors are infected with M. tuberculosis. In an attempt to ensure that as few persons as possible infected with *M. tuberculosis* will be classified as negative reactors and those not infected with *M. tuberculosis* will not be classified as positive reactors,

different cut points of measured indurations are used for different groups, depending on the risk of TB for that group. In general, a reaction is classified as positive if it is more than 5 mm for human immunodeficiency virus (HIV) infected persons or more than 10 mm in others.

1.3.2. CORRELATION WITH PROTECTION

1.3.2.1. ANIMAL MODELS

Until the fifties, a close parallelism between resistance and delayed type hypersensitivity (represented by the Mantoux test) was assumed, based almost entirely upon observations of tuberculosis in the guinea pig. This species is susceptible to fatal infections by *M. bovis* and M.tb and produces skin test reactivity and granulomatous lesions comparable with the disease in humans but not exactly the same (Wiegeshaus, 1989). Guinea pigs are more susceptible to tuberculosis than humans but they have a more similar DTH reaction to humans than mice (Griffin, 1995). Studies of the pathogenesis of TB were carried out using inbred strains of rabbits, which were either susceptible or resistant as discussed earlier. The vast majority of research using experimental animals in recent years has involved inbred mice, because of the availability of reagents necessary to study the genetic and cellular basis of immune reactivity following virulent infection.

Although many writers assumed a causal connection between the protection and skin reactivity, many researchers showed already in the past that animals could be desensitized (no skin reaction) and still remain resistant to tuberculosis (Rich, 1951). In these experiments, animals that remained in good health after having resisted a tuberculous infection could have a drop in skin reactivity to a very low level and after the lapse of one or two years even large doses of tuberculin would fail to produce any reaction. The animals which have lost their skin reactivity with the passing of time would still retain in high degree the resistance to reinfection that was conferred by the original infection. Resistance to reinfection would persist even when skin reactivity was deliberately abolished by desensitizing procedures (Rich, 1951).

A very promising animal model to study tuberculosis infection and DTH reactions is the study of *M. bovis* infections in farmed deer (Griffin, 1993) since *M. bovis* has a close phylogenetic relationship with M.tb and the pattern of disease development and DTH in deer has much similarity with that seen in humans (Griffin, 1995).

1.3.2.2. HUMANS

In the past, extensive Mantoux surveys were done to establish tuberculosis morbidity and tuberculin sensitivity. One of the best known was the study done by Palmer and colleagues (Palmer, 1957) in which nearly one hundred thousand recruits were surveyed and followed at the San Diego Naval Training Center from 1949 to 1951. This study is still the base for the current 10

mm cut off in Mantoux testing for infection which was later reinforced by studies of exposure to environmental mycobacteria (Edwards 1958, Edwards 1965, Lockwood 1987, Menzies 1992). Nevertheless, many studies had shown that the relationship between size of Mantoux reaction and disease is different in different geographic areas and sometimes difficult to establish (Nyboe 1960, Bouros 1995, Rieder 1995, Johnson 1995, Aziz 1985, Tayler 1995).

Another important use for the Mantoux test is to evaluate vaccination. It is expensive and time-consuming to assess the protective immunity imparted by mycobacterial vaccines in human populations. On the other hand it is simple to evaluate the sensitization imparted by such vaccines, at least in terms of DTH to mycobacterial skin test antigens by the Mantoux test. Because of this facility, the Mantoux test has been used as a surrogate measure of immunogenicity in vaccine development and in the monitoring of vaccine potency, and most mycobacterial vaccine trials have devoted considerable effort to pre-and post-vaccination skin testing. Its use in such context implies belief in a correlation, or at least an informative relationship between DTH and protective immunity. It is widely accepted that strong naturally acquired tuberculin sensitivity is an indicator of infection by - and of an immune response to- the tubercle bacillus, but that it is not a measure of protective immunity against clinical tuberculosis. Strong natural tuberculin sensitivity does not appear to indicate protective immunity against tuberculosis(Fine, 1986). According to Fine (Fine, 1986), some BCG trials showed that intermediate levels of tuberculin sensitivity are associated with reduced risk of TB but this is not confirmed in other populations.

The specific host immune response during active tuberculosis is still not well understood (Amara, 1996). Normally the tuberculous host develops skin hypersensitivity some 3 to 6 weeks after the initial exposure to *M. tuberculosis*, and this response may be the only indication that an infection has actually occurred. Some studies showed that loss of skin test reactivity is present in advanced stages of disease with a concomitant rise in antibody titers and these findings are reversible with clinical improvement after tuberculosis treatment (Bhatnagar 1977, Schachter 1972). In humans not only is a negative Mantoux test no proof that the individual has never been infected, but it is also no proof that acquired resistance is lacking since data from BCG trials have shown that skin reactivity after vaccination can vanish with time (Narain, 1976).

All these considerations obviously have an important bearing upon the proper interpretation of the results of Mantoux surveys. In developing countries, where the mortality and morbidity from tuberculosis are very high, and sputum-positive cases are far more numerous in the population than they are in developed countries, individuals who became infected have a greater opportunity to become infected again and again throughout their lives, and so to have their skin reactivity frequently restimulated. In developed countries, the smaller numbers of open cases leads to a decrease in the number and frequency of repeated reinfections. The value of the tuberculin testing diagnosis in adults will, of course, increase in parallel with the decrease in the incidence of infection in the population. The decrease of infection increases the number of

negative reactors, and after infancy and young childhood it is the negative test that is helpful in diagnosis.

1.3.2.3. MANTOUX AND BCG

BCG vaccine was introduced in 1921 and was standardized in 1966. Many authors refer to BCG as a failure (Grange, 1994). This is an oversimplification. There is much controversy in this matter, with many studies showing a variability of protection from 0 to 80 percent (Colditz, 1994). There are many factors accounting for this variability, such as exposure to environmental mycobacteria (Fine, 1995), BCG strains used in the studies (Brewer, 1995), and Mantoux criteria for inclusion in the studies (Baily, 1980). A detailed discussion of BCG is beyond the scope of this review. Without any doubt, the major protective effect of BCG is to confine a primary tuberculosis infection to the lungs and prevent its hematogenous spread, affording greater protection against TB meningitis or disseminated TB (Grange 1983, Wunsch-Filho 1993). Conversion to positive Mantoux after BCG vaccination has historically been used as a proxy measurement of immunization (Ciesielski, 1995). A number of studies have shown that the development of immunity and resulting positive Mantoux varies greatly with the age of the vaccinee (Ildirim, 1992). In a group of schoolchildren and young adults in Montreal, Menzies and Vissandjee (Menzies, 1992) found that just 7.9% of those BCG vaccinated in infancy had significant tuberculin reactions compared to almost 26% of the group vaccinated after 5 years of age. The immunologic response to BCG, measured in terms of antibody

levels and Mantoux reaction, decreases fairly rapidly after vaccination (O'Mahony, 1990). Data from the Madras trial (Narain, 1976) showed that nearly half the children in the age group 0 to 4 years had reverted to negative reactors (0 to 7 mm) two and half years after BCG vaccination. In a study of 149 Asian toddlers in Birmingham, it was found that nearly half of the children had a negative response 2 years after BCG vaccination (Grindulis, 1984). In this study, no child was clinically ill or overtly malnourished and none had evidence of severe deficiencies of protein, energy, iron, or zinc. These studies demonstrate that one should not be assumed to be skin test positive because of a history of past BCG vaccination.

1.3.2.4. DTH REACTION

In 1890, Robert Koch observed that individuals infected with M.tb developed a localized inflammatory response when injected subcutaneously with a filtrate derived from a mycobacterial culture. He called this localized skin reaction a "tuberculin reaction". Later, as it became apparent that a variety of other antigens could induce this response, Coombs and Gell suggested that the name be changed to DTH or hypersensitivity type 4 (Turk, 1980). It was suggested as a general category to describe all those hypersensitivity reactions that took more than 12 hours to develop.

Koch sought to exploit this phenomenon for the treatment of TB, and found that injection of larger quantities of old tuberculin subcutaneously into tuberculosis patients would evoke necrosis in established tuberculous lesions at

distant sites. This resulted in necrosis and sloughing of the lesions of skin tuberculosis but when similar necrosis was evoked in deep lesions in the spine or lungs, the results were disastrous, and merely provided further necrotic tissue in which the bacteria could proliferate. As described by Rich (Rich,1951), even with such terrible previous results many doctors started to use this treatment, but with much smaller doses of old tuberculin. It was administered in gradually increasing doses in the absence of detectable reactions. They noticed that improvement paralleled desensitization. These data was quoted by Rich (Rich, 1951) without further details. The treatment was very slow and was abandoned with the introduction of the first tuberculostatic drug in 1944. These experiments originated the idea that DTH (still called simply 'hypersensitivity' by many authors) was responsible for the development of disease following M.tb infection. This idea is still pursued by many researchers (Dannenberg 1994, Grange 1994, Rook 1994). The skin site of a DTH response (determined as foot pad swelling in animals or Mantoux reaction in humans) is often assumed to be a model of *in viscera* response and, in the field of tuberculosis, there is not strong evidence that this is always true.

Intradermal injection of neutralizing antibodies against guinea pig TNFα or human macrophage chemotactic factor (MCF-1) partially suppressed guinea pig DTH reaction, suggesting that these two cytokines were involved in its generation (Higashi, 1995). Studies in mice showed that TNFα plays an important role in necrotic reactions at DTH (skin) sites. The injection of TNFα into DTH sites evoked with soluble mycobacterial antigen in preimmunized mice

causes hemorrhagic necrosis at those sites (Al Attiyah, 1992). This finding, added to other experiments that show the direct involvement of TNFα in granuloma formation (Amiri, 1992), bring to light its connection with both tissue-damaging and protective responses. Hernandez-Pando and Rook (Hernandez-Pando, 1994), using *M. vaccae* in a murine model, suggested that the paradoxical role of TNFα is dependent on the parallel T cell cytokine pattern evoked. In this model, the absence of tissue damage upon TNFα introduction is associated with a clear TH1 response whilst mixed responses (mixed TH1 + TH2 or TH0) were associated with increased sensitivity to TNFα. TNFα may also have another role: activating the most potent antigen presenting cells, the dentritic cells. Thurnher et al. (Thurnher, 1997) showed, in an *in vitro* model, that BCG infection of human dentritic cells induced the expression of the dentritic-cell-maturation antigen CD83 and stimulated TNFα-gene transcription and TNFα-protein release from dendritic cells. When BCG infection was carried out in the presence of neutralizing anti-TNFα antibody, the expression of CD83 was inhibited by more than 50%. These findings suggest the involvement of TNFα in the maturation of dendritic cells.

Is DTH in the skin and at the site of disease exactly the same phenomenon? It is uncertain to what degree the hypersensitivity response resulting in a positive skin test and the immune response conferring resistance to the disease are similar. Although DTH and protective immunity emerge in parallel following primary tuberculosis infection, analysis of BCG trials indicates that DTH is not predictive of protection (Ellner, 1997). This observation also has

support from experimental infections in inbred mice showing that the DTH response can be dissociated from protection (Youmans 1969, Orme 1984). Orme has demonstrated that while killed mycobacterial vaccines induce significant levels of DTH in mice, they do not evoke a protective immune response (Orme, 1988). A very interesting finding came from a study done by Jensen et al. (Jensen,1977) at the Statens Seruminstitut. When studying PPD stimulated lymphocyte proliferation (LP), they found that persons with negative Mantoux and positive LP were those involved in the manufacture of PPD at the Statens Seruminstitut. This was one of the first observations from laboratory studies of human subjects questioning whether both *in vivo* and *in vitro* assays of cellular immunity are an expression of the same phenomenon, and thus can be used interchangeably, or they are completely separated events.

1.3.2.5. BOOSTING PHENOMENON

Some tuberculin reactors' sensitivity to tuberculin will decline over time and their skin test will become negative. There is no consensus on the definition and interpretation as well as the immunologic mechanism involved in a boosting reaction after sequential tuberculin testing (Menzies, 1992). Some researchers believe that the administration of a single tuberculin skin test can boost immunologic memory and a second tuberculin skin test would react positive up to 2 years later (Horowitz, 1995). Menzies (Menzies, 1994) found 5.2% of students entering health professional training programs in Montreal had booster reactions, and they were associated with older age and previous BCG

immunization. A study in the United States of Southeast Asia refugees found 31% had boosting reactions and they were associated with previous exposure to environmental mycobacteria and a history of BCG vaccination. Two step skin testing will prevent such individuals from being reported as convertors (Barnes, 1993). Upon a negative skin test, a second skin test should be placed within 1 to 4 weeks, and the result of the second test would be considered the base line (Starkey, 1995).

1.3.2.6. ENVIRONMENTAL MYCOBACTERIA

The predominant cause of a false positive reaction is hypersensitivity to environmental mycobacteria. These often ubiquitous inhabitants of soil and water are typically pathogenic only in the immunocompromised host. In endemic areas the majority of the population has been exposed to environmental mycobacteria, which are cross-reactive with *M.tuberculosis* on PPD testing. In such areas, cross reactions are likely to outnumber reactions caused by *M. tuberculosis* (Dascalopoulos, 1995). Many studies used tuberculins made from atypical mycobacteria (Edwards 1965, Lockwood 1987, Menzies 1992) in the hope of differentiating infection caused by environmental mycobacteria from tuberculosis infection. These studies were used to reinforce the 10 mm cut off for Mantoux Test positivity due to the observation that exposure to environmental mycobacteria typically would give a reaction of 3 to 8 mm. This is not so clear in other studies (Narain 1978, March-Ayuela 1990). Overall, there is no clean

separation between those infected with *M. tuberculosis* and those who are infected with environmental mycobacteria.

1.3.3. STUDIES DONE IN THE SKIN

Biopsy studies, collected at different time intervals, from Mantoux positive sites showed that, six hours after the injection of PPD, an increased number of T cells and a slightly increased number of macrophages were seen scattered in the sub-epidermal area, around blood vessels, with some in clusters (Beck, 1991). Chu et al. (Chu, 1992) showed that the number of T cells increased markedly at 24 hours and reached a peak at 48 hours, but declined at 72 hours and then remained constant until day seven. Macrophages rapidly increased from 24 to 48 hours and reached a peak at 72 hours. A few CD 4^+ T cells were found in the epidermis at 48 and 72 hours. The CD4:CD8 T cell ratio remained approximately 2:1 throughout the reaction. A slight decrease of macrophages was seen at day seven. The number of Langerhans cells increased at 24 hours in the epidermis, reached a peak of about two fold the number in normal epidermis at 48 hours, and declined thereafter. They were also seen in the upper dermis and some in cell aggregates at 48 and 72 hours, but not at other time points. B lymphocytes were not found. IFNγ staining cells were $CD3^+$ cells and declined after a peak at 48 hours, even though a significant number of T cells (about 50%) still persist in the lesion. TNFα staining cells were macrophages and persisted throughout the seven days. Using the skin window

chamber technique, MacPhee et al. (MacPhee, 1993) recovered T cells from PPD and Candida reactions. They established T cell clones in limiting dilution cultures and found at least 26 -fold enrichment of antigen-specific cells in skin window chamber over blood.

1.4. CYTOKINES AND PROTECTION

1.4.1. THE TH1/TH2 MODEL

Studies from murine $CD4^+$ T cell-clones described the existence of at least two functionally distinct subsets of cells: TH1 cells that secrete interleukin 2 (IL2) and interferon gamma (IFNγ) upon activation but not interleukin 4 (IL4) or interleukin 5 (IL5), and TH2 cells that produce IL4 and IL5 but not IL2 or IFNγ (Mosmann 1986, Cherwinski 1987). They are also functionally distinct -- TH1 cells mediate a DTH response (Cher, 1987) and although TH2 cells can sometimes mediate an inflammatory reaction (Chensue 1994, Chensue 1995), they are more effective at inducing allergic reactions and providing help for antibody production by B cells (Coffman, 1986). The cytokine patterns of other T cell subsets are still being defined as well as other cells of the immune system with TH1 or TH2-like cytokine pattern (Mosmann, 1996). In addition, TH1 and TH2 cell types appear to regulate each other via their cytokine synthesis (Sher, 1992). Experiments with transgenic mice showed that the development of a TH1

or TH2 response is primarily influenced by the cytokine environment during the initial phase of immune responses (Hsieh 1993, Seder 1992, Swain 1990).

1.4.2. CYTOKINES AND OTHER IMMUNE PARAMETERS DURING TUBERCULOSIS

Classic animal studies done by Mackaness in the sixties already showed that the acquired cellular immune response to TB consists of T cells and mononuclear phagocytes, which act in concert to control the bacterial infection. Recent studies have added more information and have also modified this traditional model. Microbial antigens are processed by macrophages and presented in the context of gene products of the major histocompatibility complex which stimulate antigen-specific T- lymphocytes. The activated T-cells differentiate and produce cytokines which ultimately induce macrophage activation. Macrophages infected with M.tb or exposed to mycobacterial products produce cytokines such as TNFα, IL12, IL1 and IL6 (Orme, 1993). $CD4^+$ T cells are traditionally thought to be the primary T cell subset responsible for regulating the human immune response to mycobaterial infection (Kaufmann, 1993). However, studies in man and animal models have demonstrated that $CD8^+$T cells and $\gamma\delta$ T cells are also involved. $\gamma\delta$ T cells are activated by mycobacteria in vitro and accumulate in sites of mycobacterial infection *in vivo* (Tsukaguchi,1995). $CD8^+$ T cells are crucial for protection in the mouse model,

as animals lacking the gene for either β2-microglobulin or CD8 die of experimental TB infection (Flynn, 1992).

Current information about the role of cytokines in host defense against tuberculosis is not well defined due to conflicting data obtained from patients, and studies in experimental animals and *in vitro* models. Human T cell clone studies suggest that both TH1 and TH2 responses can develop independently within the same individual (Del Prete1991, Sartono1996). IFNγ is an important immunoregulatory cytokine which has been shown *in vitro* to enhance killing of several intracellular pathogens such as *Legionella sp* (Bhardwaj,1986), *Leishmania sp* (Murray,1983) and *Chlamydia sp* (Rothermel, 1983); however, its ability to stimulate anti-tuberculous activity in human macrophages is still not proven. Douvas and colleagues (Douvas, 1985) described enhanced mycobacterial replication in human macrophages pretreated with IFNγ, when compared with untreated macrophages. This finding was supported by Rook and colleagues (Rook, 1986), who found IFNγ treated human macrophages were permissive of mycobacterial growth. The opposite was found in murine bone marrow macrophages (Flesch, 1987) and in murine peritoneal macrophages (Rook, 1986). Denis (Denis, 1991) reported that infusion of recombinant IFNγ led to improved survival and decreased viable bacterial organ counts in Balb/c mice infected with M.tb. Furthermore, treatment of infected mice with anti-IFNγ antibody decreased survival and prevented the development of protective immunity in IFNγ-treated infected mice. The differences observed when comparative studies were carried out with mouse macrophages and human

macrophages could be related to the inability of human macrophages to produce nitric oxide when stimulated by IFNγ or TNFα. Recently, Tascon et al. (Tascon, 1998), transferring CD8 T cells from normal and IFNγ knockout mice to M.tb infected athymic mice, showed the importance of IFNγ since protection was associated with transfer of cells from normal mice. In humans, there are reports of increased susceptibility to mycobacterial infection in three children that lack IFNγ receptor 1 (Newport 1996, Jouanguy 1996). In addition, in humans the presence of other substances such as calcitriol have been shown to be important for macrophage activation (Rook 1989, Denis1991).

Surcel et al. (Surcel 1994), analyzing PBMC response to two mycobacterial antigens from TB patients, concluded that there is no deficient IFNγ production in active tuberculosis. However, Zhang et al. (Zhang,1995) found that there is depressed IFNγ production. They compared IFNγ, IL2, IL4, IL10 and IL13 production and mRNA expression from live M.tb-stimulated peripheral blood mononuclear cells between TB patients and healthy tuberculin reactors. They found that pulmonary tuberculosis was associated with a depressed TH1 but not an increased TH2 response. They also found that IFNγ production increased with therapy and IL10 concentrations did not change significantly. Since immunologic events are not always reflected in the peripheral blood, Lin et al. (Lin, 1996) analyzed lymph nodes from patients with tuberculous lymphadenitis. They showed that in contrast to the depressed TH1 response of *M. tuberculosis*-stimulated PBMC, the local immune response in TB is characterized by enhanced T cell production of IFNγ. They also confirmed an

absence of an enhanced TH2 response in tuberculosis. Taha et al. (Taha,1997), when examining bronchoalveolar lavage (BAL) cells, found an increased number of $CD8^+$ cells and increased numbers of IL12 and IFNγ mRNA-positive cells in the BAL of patients with active as compared to inactive tuberculosis. A very interesting observation was recently shown by Dlugovitzky et al. (Dlugovitzky,1997). When classifying TB patients according to their pulmonary lesions by X-ray findings, they found that serum IL2 and IFNγ were higher in those patients with mild and moderate disease while IL4 was higher in those with moderate and advanced disease.

Since TB pleuritis commonly resolves without therapy, many researchers analyzed the pleural fluid assuming that cytokines which are concentrated in the pleural space are likely to play a role in immune resistance. Studies in humans have shown that patients with TB pleuritis have high levels of TNFα and IFNγ in the pleural fluid, and pleural monocytes of infected patients have been shown to secrete high levels of these cytokines when compared to peripheral monocytes (Barnes, 1990). Il12, a cytokine that favors the development of TH1 cells and enhances cytotoxic responses, was also found in elevated concentrations at the site of disease in tuberculous pleuritis (Zhang, 1994). Elevated IL-2 soluble receptor (IL2-sr) levels have been described in TB pleural effusions and in the sera of patients with active TB (Champsi, 1994). It has been proposed that IL2-sr may have a role in pathogenesis of disease by binding IL2 in the sera and preventing it from interacting with cellular targets, thereby causing depressed

cellular immunity leading to mycobacterial disease. However, the immunologic significance of high levels of IL2-sr in tuberculosis is currently unknown.

In summary, the TH1 response appears to be depressed in tuberculosis and there is not enough evidence of a enhanced TH2 response. IFNγ appears to be associated with the immune response to M.tb but the evidence of its involvement is still controversial.

1.4.3. RELATIONSHIP BETWEEN MANTOUX STATUS AND CYTOKINES AND OTHER IMMUNE PARAMETERS

Old Tuberculin was known to have a sensitizing effect by itself (Rich, 1951). The development of PPD by Siebert was very important for the widespread use of the Mantoux reaction. The Mantoux reaction is used as a tool for TB diagnosis and to assess vaccine efficacy even though there are important considerations and doubts about that, as discussed earlier. Reports of Mantoux surveys done on a repetitive basis in medical personnel add controversy to this subject and suggest that PPD, in this context, can also have a sensitizing effect (Jensen 1977, Horowitz 1995).

Many attempts have been made to isolate an immunodominant antigen that could be used as an *in vitro* marker for disease and protection or used *in vivo* as a new vaccine to TB (Andersen, 1997). Since the relation between Mantoux reaction and protection is not reliable and we still do not know the immunologic significance of such new antigens, comparison of its use *in vitro* with the Mantoux status in the same individual does not seem very useful.

Nevertheless, because of a lack of better parameters of *in vivo* protection, this is still being done by many researchers.

The value of the lymphoproliferative (LP) response itself and its association with Mantoux is not clear, although some researchers still believe an association between skin test reactivity and *in vitro* PPD lymphocyte proliferation exists (Havlir 1991, Ellner 1996). Past studies already suggested that this is not always the case (Jensen 1977, Grindulis 1984). Schoel and Kaufmann (Schoel, 1992) analyzed LP response of human T cells to 400 antigen fractions of M.tb. They found that stimulation profiles of Mantoux positive individuals are very similar to TB patients although two Mantoux positive individuals, from a group of eight individuals, had an *in vitro* profile similar to the Mantoux negative group. The Mantoux negative group (with 11 individuals) had a pattern distinct from that of the positive group with exception of one individual who was similar to the Mantoux positive group. It was assumed by the researchers that the higher sensitivity of the *in vitro* system than of skin testing identified this person as being immune to mycobacteria.

Overall, there is a significant lack of accurate information about the Mantoux reaction and immune parameters. Over time, *in vitro* studies were usually developed to evaluate vaccine efficacy, as discussed earlier, and the Mantoux reaction was just an additional information in this context. Although new research on the immunology of M.tb infection includes novel techniques, immunologic study of the Mantoux reaction so far is limited to quantitative analysis at the site of the reaction. Most of the time, tuberculin reactors are used

as controls when evaluating any immune parameter in tuberculous patients. Since the Mantoux reaction is used widely and doubts still surround its significance, more detailed studies of its effect on the immune system are necessary.

1.5. OBJECTIVES

- DEFINE OPTIMAL CONDITIONS FOR *IN VITRO* CYTOKINE PRODUCTION
- ESTABLISH IF THE MANTOUX TEST CAUSES ANY ALTERATION IN CYTOKINE PRODUCTION AND LYMPHOCYTE PROLIFERATION
- EXPLORE A POSSIBLE CORRELATION BETWEEN THE IMMUNE PARAMETERS
- EXPLORE A POSSIBLE CORRELATION BETWEEN IMMUNE PARAMETERS AND CLINICAL STATUS

2. MATERIALS AND METHODS

Blood was drawn before and 48 hours after a Mantoux test from fifteen subjects in an ongoing Mantoux survey study of personnel at University of Alberta Hospital. The test was read and recorded by an experienced public health nurse. The test was performed by injecting 0.1 ml containing 5 tuberculin units of purified protein derivative (PPD) (Connaught laboratories) of tuberculin into the volar aspect of the forearm. Tests were read at 48 hours and any induration was measured along the diameters transverse and parallel to the long axis of the forearm. The induration size and any other reaction were recorded. No subjects were known to be immunocompromised or infected with human immunodeficiency virus (HIV) or have any other illness. 10 to 40 ml of blood was drawn (according to availability) immediately before application of PPD and 48 hours later, at reading. The blood was collected in vacutainer tubes with sodium heparin and immediately processed for the laboratory work.

2.1. ISOLATION OF LYMPHOCYTES

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by gradient sedimentation. Blood samples were centrifuged (Beckman GPKR centrifuge) at 1350 rpm (400 x g), for 10 minutes, at room temperature (RT). Plasma was collected and the cells were diluted 1:2 with RPMI. Fifteen milliliters (mL) of Ficoll - Paque (Pharmacia Biotech, Sweden) was added to 50 ml polypropylene tubes (Falcon). The diluted sample was laid carefully over the Ficoll, up to 50 mL. The tubes were centrifuged at 1350 rpm for 30 minutes, RT. The upper layer was aspirated and discarded. Using a Pasteur pipette, the

interlayer was collected and transferred to 50 mL tubes. At least three volumes of RPMI were added to the transferred layer, mixed gently and centrifuged at 1350 rpm for 10 minutes, 4^0C. The supernatant was aspirated, 40 mL of RPMI was added to the pellet, mixed gently and centrifuged at 1350 rpm for 10 minutes, 4^0C. The pellet was resuspended in tissue culture medium, the cells were counted and the volume adjusted for the desired cell concentration. Cells were counted in a hemocytometer and viability estimated using the trypan blue exclusion method. Viability was always above 95%.

2.2. TISSUE CULTURE ASSAYS

Cells were cultured at $6x10^4$ (PHA cultures for TI assays) and $2x10^5$ (all other cultures) cells in 200 µl per well in triplicate. They were left unstimulated (control cultures) or were stimulated with the antigens described in 2.2.4. Tissue culture medium consisted of RPMI 1640 with 10% heat-inactivated human serum (Sigma H-2520, lot 055H0851, from male AB plasma, LPS tested) supplemented with penicillin 50 units/ml, streptomycin 50 µg/ml (GIBCO BRL-1002) and HEPES [N-2-hydroxyethylpiperazine-N' - 2- ethanesulfonic acid) buffer 20 mM. The cell cultures were incubated at 37 ^{0}C, 5% CO_2 in a humidified incubator.

2.2.1. PROLIFERATION ASSAYS

Cells were isolated and cultured in the conditions described above. They were incubated for 4 (PHA cultures) or 6 (antigen cultures) days in 96 well cell culture flat-bottom plates (Costar, Cambridge, MA). Twenty-two hours before termination of the cultures 1µCi [^{3}H] thymidine (specific activity 2.0 Ci/mmol,

ICN Biochemicals) was added per well. Cultures were harvested with a Packard Micromate 196 cell harvester and tritium uptake was measured using a gas scintillation counter (Matrix$^{TM}_{96}$ Direct Beta Counter).

Results were expressed in counts per minute (cpm) as the geometric mean of the triplicate cultures. Stimulation was estimated as the difference between stimulated culture and unstimulated culture (control):

Δcpm = cpm of stimulated culture - cpm of control cultures

2.2.2. CYTOKINE PRODUCTION

Cells were isolated and cultured in the conditions described in 2.2, using 96 well cell culture round bottom plates (Costar, Cambridge, MA). Cultures were set up for 24 (day 1), 48 (day 2), 96 (day4) and 144 (day 6) hours. At each time point supernatants were harvested (150 to 180 µl/well) and stored at -70°C until analyzed by enzyme-linked immunosorbent assay (ELISA).

Cytokine production was estimated as the geometric mean of the triplicate cultures. The results were expressed as the difference between stimulated cultures and control cultures.

2.2.3. ANTIGENS

PURIFIED PROTEIN DERIVATIVE (PPD):

PPD without preservative, 2 mg/mL, was bought from Connaught Laboratories (Willowdale, ON) and stored at 4°C. Three concentrations of PPD

were tested-- 2, 10 and 50 μg/mL. These dilutions were made with tissue culture medium and kept at 4°C for 7 days. After this period, they were discarded and a new dilution would be made.

TETANUS TOXOID PURIFIED (TT):

TT, 2500 Limes flocculation (Lf)/mL, was bought from Connaught Laboratories and stored at 4°C. It was diluted with tissue culture medium and used at 25 Lf/mL.

PHYTOHEMAGGLUTININ (PHA-P):

PHA-P (Sigma, catalog number L-9132) is a mixture of glycoproteins from red kidney beans. It has mitogenic and agglutinating properties. The lyophilized powder was reconstituted with tissue culture medium to a concentration of 1 mg/mL, aliquotted in 1 ml vials and kept at -20 °C. When needed, one vial would be thawed and immediately used. Further dilution was made with tissue culture medium to a final concentration of 2 and 10 μg/mL. The leftover was discarded.

CANDIDA EXTRACT:

It was bought as a bulk extract (Hollister-Stier, Miles Canada Inc.) at 1: 10w/v concentration. The final concentration used in the culture was 1:100 w/v.

2.3. ELISAs

Heterosandwich ELISAs were developed to measure the amount of IFNγ, TNFα, IL4, IL5 and IL13 in the cell cultures supernatants.

2.3.1. REAGENTS

TMB SOLUTION

100 µl TMB (3, 3, 5, 5 - tetramethyl-benzidine, Sigma T-2885) in DMSO (10 mg/ml)

0.5 ml diluent buffer (NaAc 2 M pH 5.5)

15 µl H_2O_2 (30% w/v)

9.4 ml H_2O

ABTS SOLUTION

25 mg ABTS (2,2 - Azino-Bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt, Sigma A1888)

5.5 ml Na_2HPO_4 0.2 M

7 ml citric acid 0.1 M

2.5 µl H_2o_2 (30% w/v)

2.6 12.5 ml H_2O

STOP SOLUTION

H_2SO_4 1.8 N, diluted 1:20 in double distilled water.

2.3.2. GENERAL ELISA PROCEDURE

A covered plastic container, lined with wet paper towels, was used in all the incubation steps that were done at room temperature (RT). ELISA Easy Wash plates (Corning) were coated with first antibody (diluted in PBS) 50 µl/ well overnight at 4° C. Blocking solution (1% BSA in PBS, pH 7.4), 100 µl/well, was added in the morning and kept for 30 minutes at room temperature (RT). The plates were washed twice with PBS plus 0.05% tween 20 (PBST). The standard (recombinant cytokine) and samples were added, 50 µl/well, for 1 to 3 hours at RT, depending on the assay. Standards were diluted with the same medium used for tissue culture. The standard curves, with exception of the IL4 assay, started at 1000 pg/ml with doubling dilutions down to 15.6 pg/ml. Standard curves for the IL4 assay started at 500 pg/ml with doubling dilutions down to 7.8 pg/ml. Further dilution with tissue culture medium was done for day 4 and day 6 supernatants from PPD (1:3) and PHA (1:1000) stimulated cultures. Due to the small supernatant volume generated by the overall cultures (harvested 150 to 180 µl/well), samples for the IL13 and TNFα assays were transferred from IFNγ and IL5 ELISA plates respectively. The plates were then washed twice with PBST and incubated, at RT, for 1 to 3 hours (depending on the assay), with 50 µl/well of biotinylated second antibody (diluted in 1%BSA/PBST). They were washed again and then incubated with peroxidase-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:5000 in 1%BSA/PBST, 50 µl/well, for 30 minutes at RT. After a final wash, the plates were incubated with 50 µl/well of TMB solution at RT, in the dark, and stop solution, 50 µl/well, was added after 20 minutes. The absorbance was

determined at 450/490 nm with a Emax Microplate Reader (Molecular Devices, Palo Alto, CA). The sensitivity of the assays was defined by the lower limit of detection, i. e., the analyte concentration obtained from the average of the blanks plus two times their standard deviations (LLD= 0_{blanks}+ 2x SD_{blanks}). Blanks were defined as 4 wells with just tissue culture medium (no standards or samples were added). The standard curve was done in duplicate and the fitting of the curve was plotted as a quadratic function.

2.3.3. ASSAY OPTIMIZATION

The assays were optimized to make them as precise, fast and cheap as possible. Different parameters were evaluated. Optimizations were done with standards. The optimization steps were done in the same order as described.

2.3.3.1. ELISA plates:

Three different brands of ELISA plates were tested and compared in terms of highest optical density (O.D.) achieved, proximity of O.D. values between duplicates or triplicates and lower background. An IFNγ assay (as described in 2.3.2) was used. Concentration of first Ab was 1.5 μg/mL and second Ab was 0.5 μg/mL. Incubation period for standard and second Ab was two hours. Antibodies and standards were from Biosource. The results are shown in Fig. 2-1. Corning plates were considered the best.

2.3.3.2. Volume of samples/standards

Due to the small volume of supernatant available to be tested, I decided to compare ELISAS using 25 or 50 μl of standard. An IFNγ assay (as described in 2.3.2) was used. Concentration of first Ab was 2 μg/mL and second Ab was 0.5 μg/mL. Antibodies and standards were from Biosource. As demonstrated in Fig. 2-2, there is no curve with the smaller volume. A possible explanation would be that 25 μl is not enough to cover all the surface at the bottom of the wells.

2.3.3.3. Comparison between substrates:

Two different substrates were compared, ABTS and TMB. They were compared in the same ELISA plate and the experiment was repeated in three more plates. An IFNγ assay (as described in 2.3.2) was used. Concentration of first Ab was 2 μg/mL and second Ab was 0.5 μg/mL. Three points were tested, 1000 pg/mL, 500 pg/mL and background (medium). Antibodies and standards were from Genzyme. Second antibody was conjugated with horseradish peroxidase. There were three standard curves for each substrate. Results are shown in Fig. 2-3. TMB was the best substrate.

2.3.3.4. Temperature:

An IFNγ assay (as described in 2.3.2) was used to compare incubation at 20° C (RT) or 37°C (humidified incubator) during blocking, incubation with standard and incubation with second Ab at the same time. Concentration of first Ab was 2 μg/mL and second Ab was 0.5 μg/mL. Antibodies and standards were from Biosource. Two ELISA plates were set up, one for each condition. Each

plate had three points tested -- 1000 pg/mL, 500 pg/mL and background (medium) -- in triplicates. The results are shown in Fig.2-4. Since there was higher background in the assay at 37°C, I decided for incubation at RT and also because this would be easier when doing several ELISA plates at the same time.

2.3.3.5. Manufacturer:

IL 4 and IFNγ human antidodies and their recombinant cytokines(standards) were first bought from Genzyme. The assays were optimized and were working well. After a period of two months, the O.D. values dropped for both assays without an apparent reasonable explanation. Due to a time constraint, I decided to change the manufacturer rather than do any further experiment to define the reason for such change. IL4 and IFNγ antibodies and standards were then bought from Biosource European SA. Comparing IL4 standard from Genzyme and from Biosource, using for both assays Biosource antibodies, I found that their optical densities were practically the same as shown in Fig.2-5. Since I had a large amount of IL4 standard from Genzyme, I decided to continue using it.

2.3.3.6. Amount of first and second antibody:

- IFNγ ASSAY : Antibodies and standard were bought from Biosource European SA.. Three different concentrations of coating antibody were tested (as described in 2.3.2.) combined with three different concentrations of second Ab, in the same ELISA plate. Incubation time for standard and

second Ab was 2 hours. Results are shown in Fig. 2-6. The combination A/E (1μg/mL for first antibody and 0.05μg/mL for second antibody) was chosen.

- IL4 ASSAY: Antibodies were bought from Biosource European SA. and standard was from Genzyme. Four different concentrations of coating antibody were tested (as described in 2.3.2.) combined with three different concentrations of second Ab, on the same ELISA plate. Incubation time for standard and second Ab was 2 hours. The higher background seen with the higher amount of second antibody could be due to nonspecific binding to the solid phase or to some technical error but this does not affect the antibody concentration chosen. Results are shown in Fig. 2-7. The combination D/G (0.25μg/mL for first antibody and 0.025μg/mL for second antibody) was chosen.
- IL5 ASSAY: Antibodies and standard were bought from PharMingen. Three different concentrations of coating antibody were tested (as described in 2.3.2.) combined with three different concentrations of second Ab, on the same ELISA plate. Incubation time for standard and second Ab was 2 hours. Results are shown in Fig. 2-8. The combination C/c (0.05μg/mL for first and second antibody) was chosen.
- IL13 ASSAY: Antibodies and standard were bought from PharMingen. Three different concentrations of coating antibody were tested (as described in 2.3.2.) combined with three different concentrations of second Ab, on the same ELISA plate. Incubation time for standard and second Ab was 3 hours

(as recommended by the manufacturer). Results are shown in Fig. 2-9. The combination A/a (1μg/mL for first and second antibody) was chosen.

- TNFα assay: Antibodies and standard were bought from PharMingen. Three different concentrations of coating antibody were tested (as described in 2.3.2.) combined with three different concentrations of second Ab, on the same ELISA plate. Incubation time for standard and second Ab was 3 hours (as recommended by the manufacturer). Results are shown in Fig. 2-10. The combination B/a (0.5μg/mL for first antibody and 1μg/mL for second antibody) was chosen.

2.3.3.7. Incubation time for the standard and second antibody:

- IFNγ ASSAY: Two incubation periods were tested simultaneously for standard and second antibody, one and two hours. Results are shown in Fig. 2-11. The shorter incubation time was chosen.
- IL4 ASSAY: Two incubation periods were tested simultaneously for standard and second antibody, one and two hours. Results are shown in Fig. 2-12. The shorter incubation time was chosen.
- IL5 ASSAY: Two incubation periods were tested simultaneously for standard and second antibody, one and two hours. Results are shown in Fig. 2-13. The shorter incubation time was chosen.
- IL13 ASSAY: Three incubation periods were tested simultaneously for standard and second antibody - one, two and three hours. Results are shown

in Fig. 2-14. Since there was a marked drop in O.D. values with the shorter incubation times, the three hours incubation period was chosen.

- TNFα ASSAY: Three incubation periods were tested simultaneously for standard and second antibody - one, two and three hours. Results are shown in Fig.2-15. Since there was a marked drop in O.D. values with the shorter incubation times, the three hours incubation period was chosen.

2.3.4. FINAL FORMAT

2.3.4.1. IFNγ ASSAY

The first antibody (Ab) and the biotinylated second Ab were monoclonal antibodies purchased from Biosource European SA. They were aliquotted and kept at -70 °C. They were used at a concentration of 1μg/mL for the first Ab and 0.05 μg/mL for the second Ab. The recombinant human IFNγ (standard) was also from Biosource European SA. The lyophilized IFNγ was reconstituted at 100 ng/mL with PBS/10% FCS, aliquotted and, as recommended by the manufacturer, stored at -70°C. Incubation for standards/samples and second Ab was done at RT, for one hour. LLD was 30 pg/mL.

2.3.4.2. IL4 ASSAY

The first antibody and the biotinylated second Ab were monoclonal antibodies purchased from Biosource European SA. They were aliquotted and kept at -70 ° C. They were used at a concentration of 0.25μg/mL for the first Ab

and 0.025 μg/mL for the second Ab. The recombinant human IL4 (standard) was from Genzyme Diagnostics. Incubation for standards/samples and second Ab was done at RT, for one hour. LLD was 30 pg/mL. The lyophilized IL4 was reconstituted at 100 ng/ml with RPMI/10% FCS, frozen in aliquots and, as recommended by the manufacturer, stored at -70°C.

2.3.4.3. IL5 ASSAY

The first antibody and the biotinylated second Ab were monoclonal antibodies purchased from PharMingen. They were aliquotted and kept at -70 ^{0} C. Both Abs were used at a concentration of 0.05μg/ml. Incubation of standards/samples and second Ab was done at RT, for one hour. LLD was 15 pg/mL. The recombinant human IL5 (standard) was also from PharMingen. The original stock was diluted with PBS/10% FCS at a final concentration of 100 ng/ml, frozen in aliquots and, as recommended by the manufacturer, stored at -70°C.

2.3.4.4. IL13 ASSAY

The first Ab was a monoclonal Ab and the second was a biotinylated polyclonal Ab (both from PharMingen). They were stored at 4^{0}C and were both used in a concentration of 1μg/mL. Incubation for standards/samples and second Ab was done at RT, for three hours. LLD was 30 pg/mL. The recombinant human IL13 (standard) was also from PharMingen. The original

stock was diluted with PBS/10% FCS at a final concentration of 100 ng/ml, aliquotted and, as recommended by the manufacturer, stored at -70°C.

2.3.4.5 TNFα ASSAY

The first antibody and the biotinylated second Ab were monoclonal antibodies purchased from PharMingen. They were stored at 4^0 C. They were used on a concentration of 0.5μg/mL for the first Ab and 1 μg/mL for the second Ab. Incubation for standards/samples and second Ab was done at RT, for three hours. LLD was 30 pg/mL. The recombinant human TNFα (standard) was also from PharMingen. The original stock was diluted with PBS/10% FCS at a final concentration of 100 ng/ml, aliquotted and, as recommended by the manufacturer, stored at -70°C.

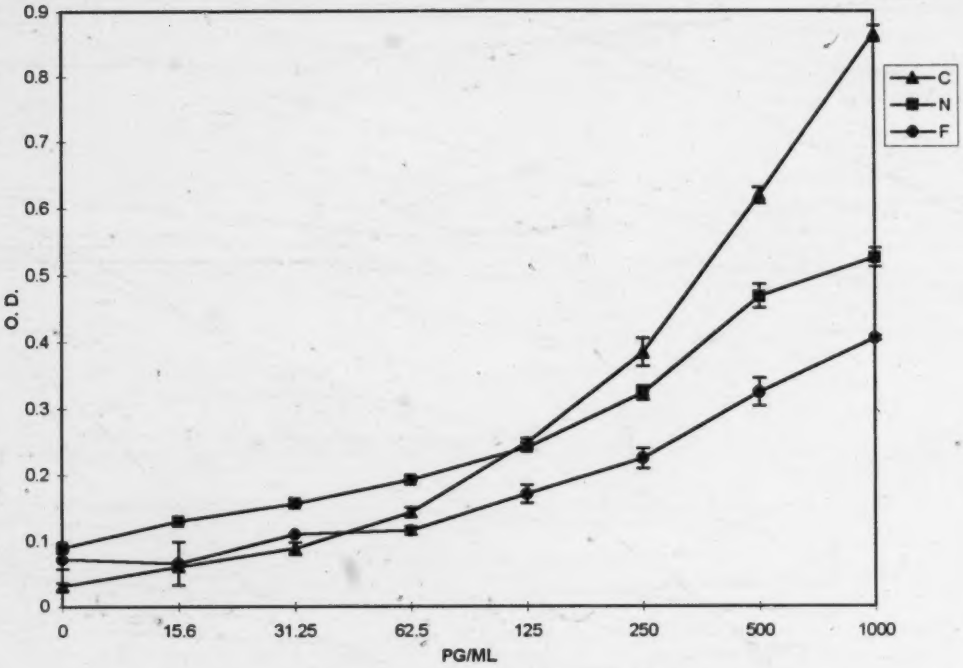


Figure 2-1. IFNγ assay - testing three different brands of ELISA plates: Corning (C), Maxisorb Nunc (N) and Falcon soft plates (F). The error bars are the standard deviation of four replicates.

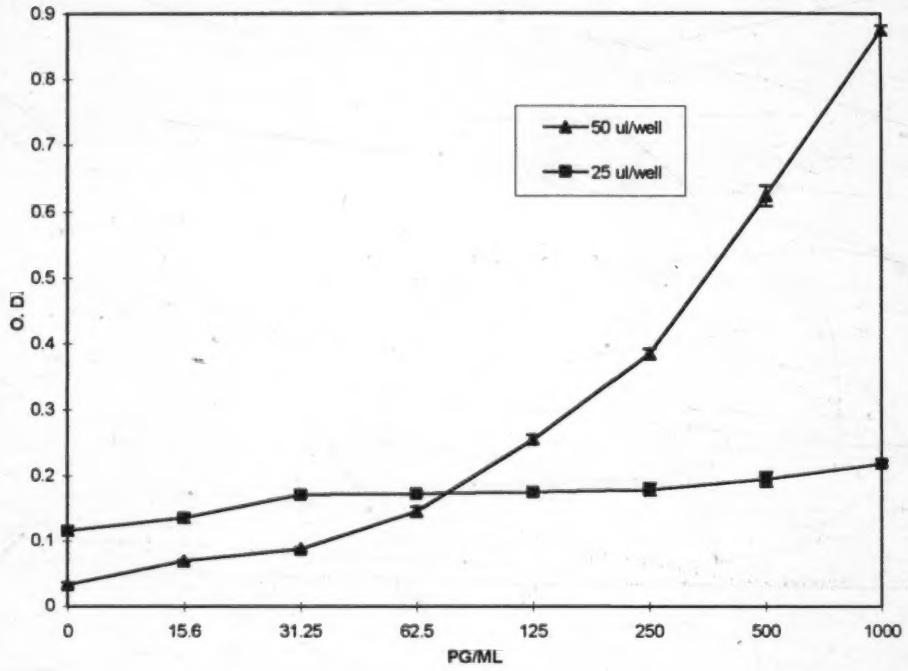


Figure 2-2. IFNγ assay - testing two different volumes of standard. The error bars are the standard deviation of triplicates.

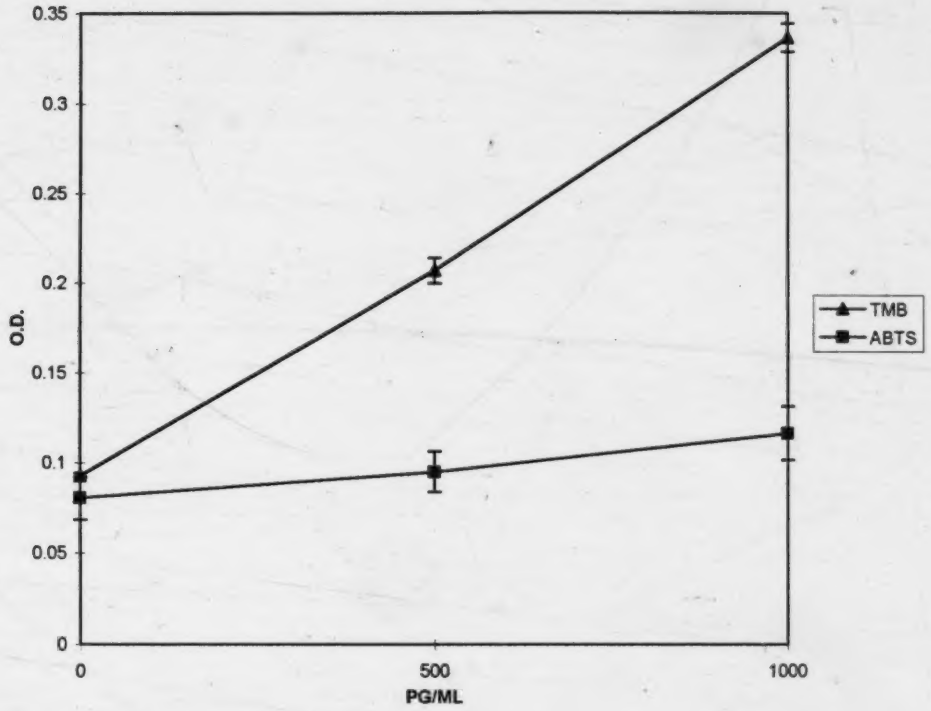


Figure 2-3. IFNγ assay (Genzyme) - testing two different substrates, TMB and ABTS. The error bars are the standard deviation of triplicates.

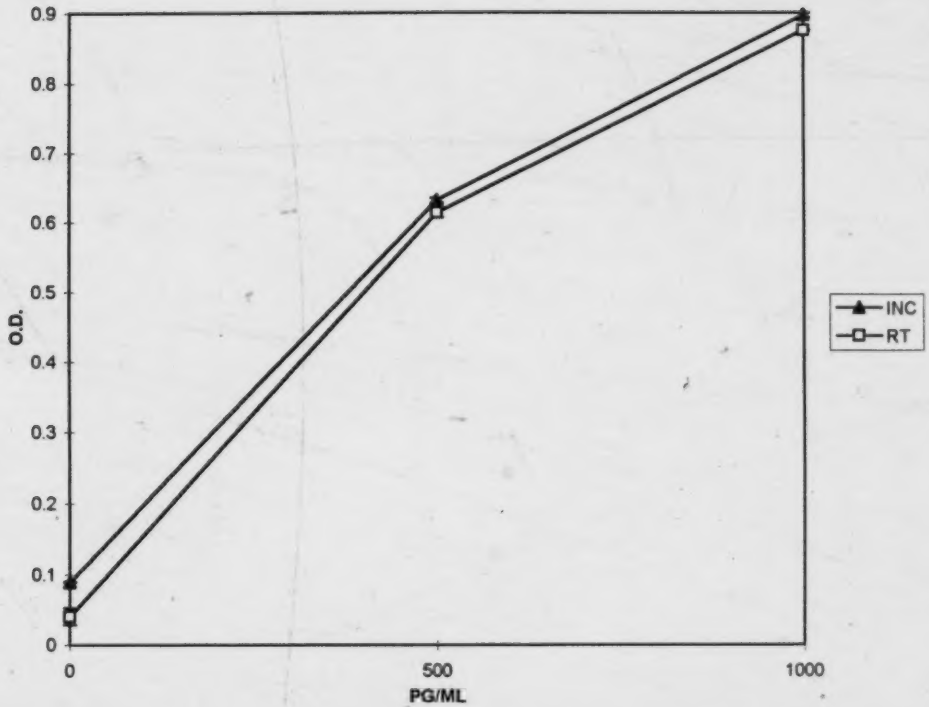


Figure 2-4. IFNγ assay - testing different incubation temperatures, 20°C at room temperature (RT) and 37°C in a humidified incubator (INC). The error bars are the standard deviation of triplicates

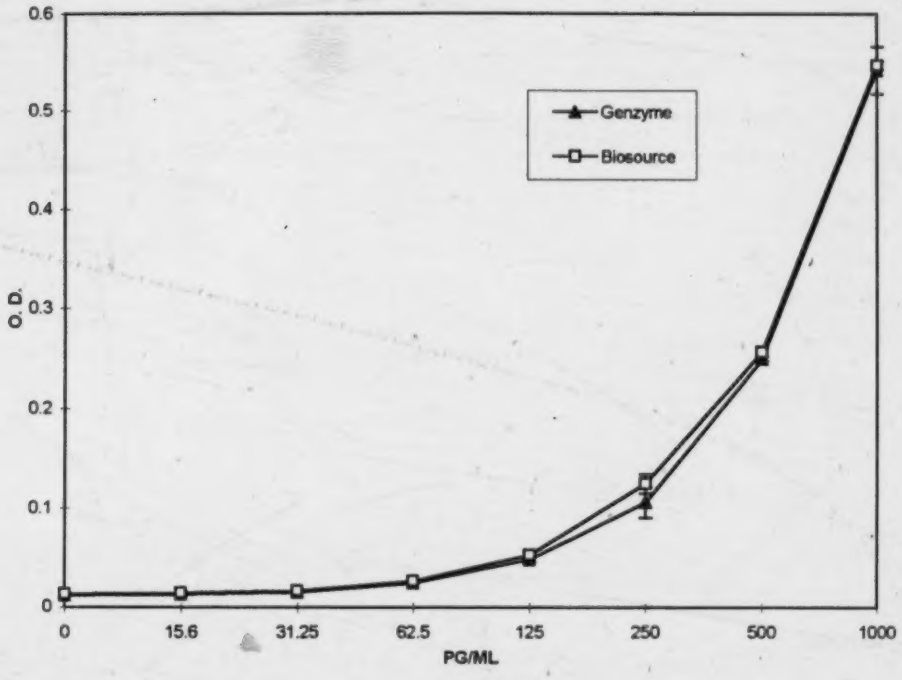


Figure 2-5. IL4 assay - testing standard from different manufacturers, Genzyme and Biosource. The error bars are the standard deviation of triplicates.

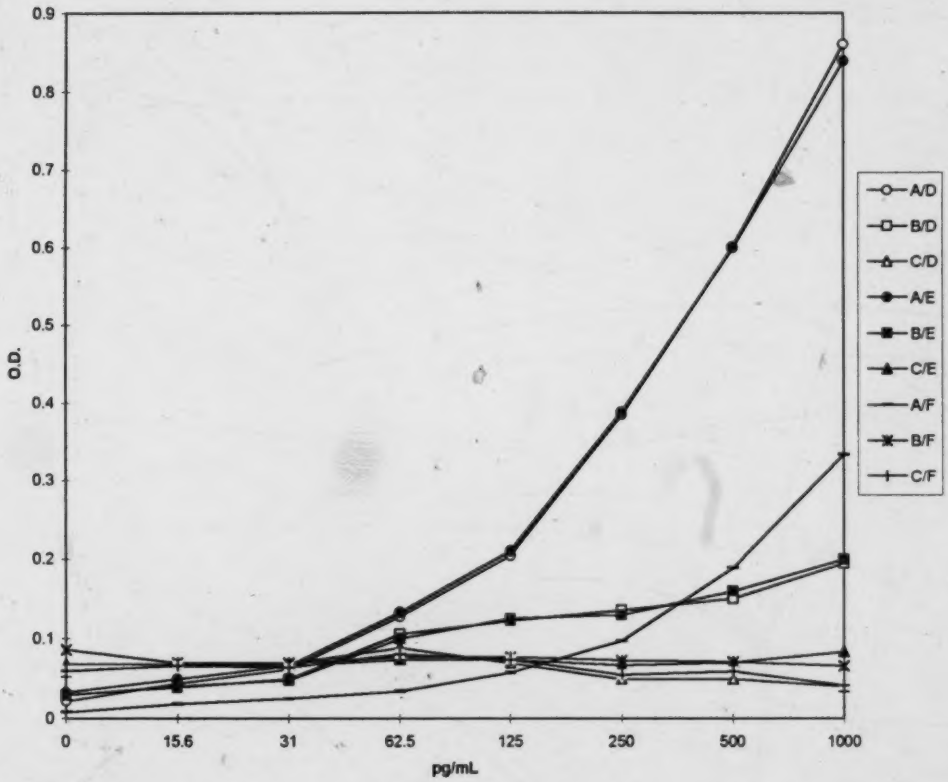


Figure 2-6. IFNγ assay - testing three different concentrations of first antibody combined with three different concentrations of second antibody. Legend: letters represent combined concentrations of first antibody (A=1 μg/ml, B=0.5 μg/ml, C=0.25 μg/ml) and second antibody (D=0.1 μg/ml, E=0.05 μg/ml, F=0.025 μg/ml).

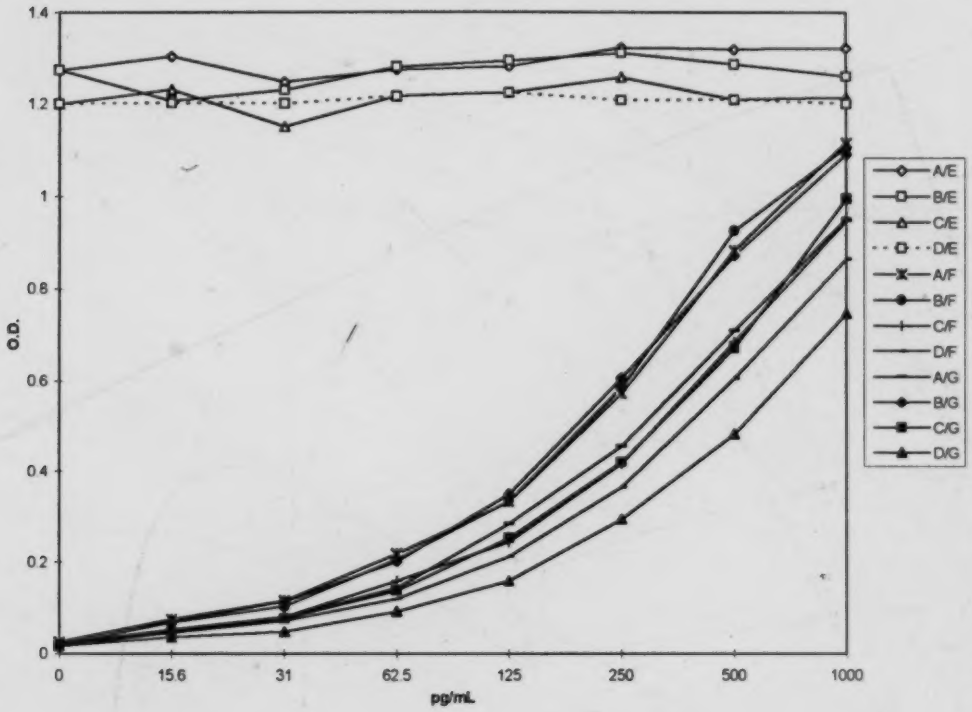


Figure 2-7. IL4 assay - testing four different concentrations of first antibody combined with three different concentrations of second antibody. Legend: letters represent combined concentrations of first antibody (A=2 μg/ml, B=1 μg/ml, C=0.5 μg/ml, D=0.25 μg/ml) and second antibody (E=0.1 μg/ml, F=0.05 μg/ml, G=0.025 μg/ml).

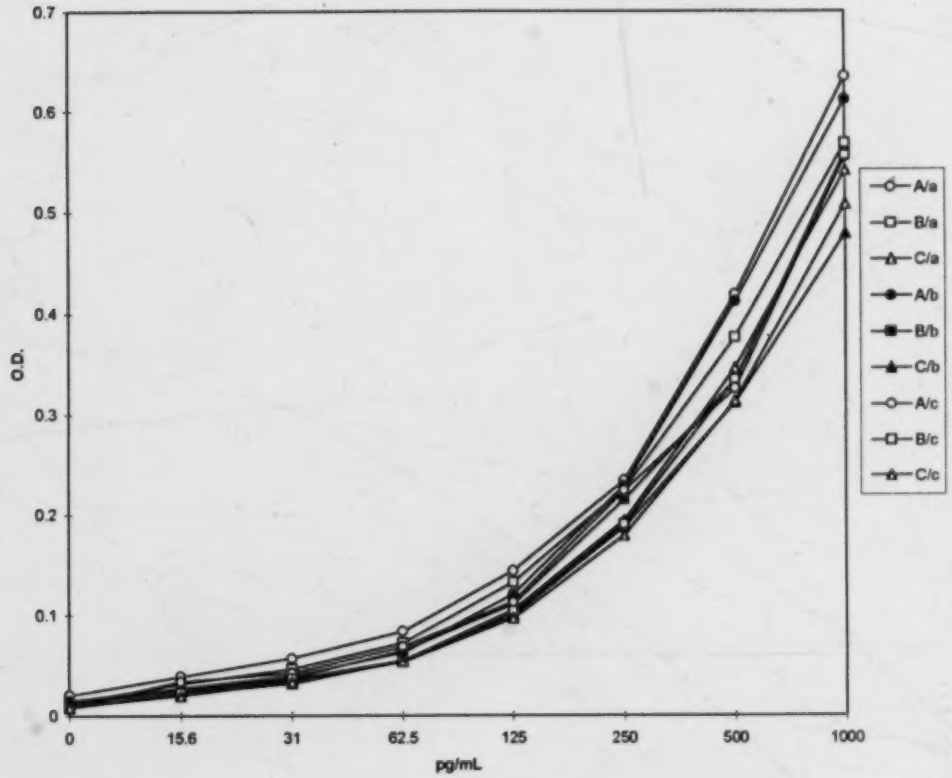


Figure 2-8. IL5 assay - testing three different concentrations of first antibody combined with three different concentrations of second antibody. Legend: letters represent combined concentrations of first antibody (a=0.5 μg/ml, B=0.125 μg/ml, C=0.05 μg/ml) and second antibody (a=0.5 μg/ml, b=0.125 μg/ml, c=0.05 μg/ml).

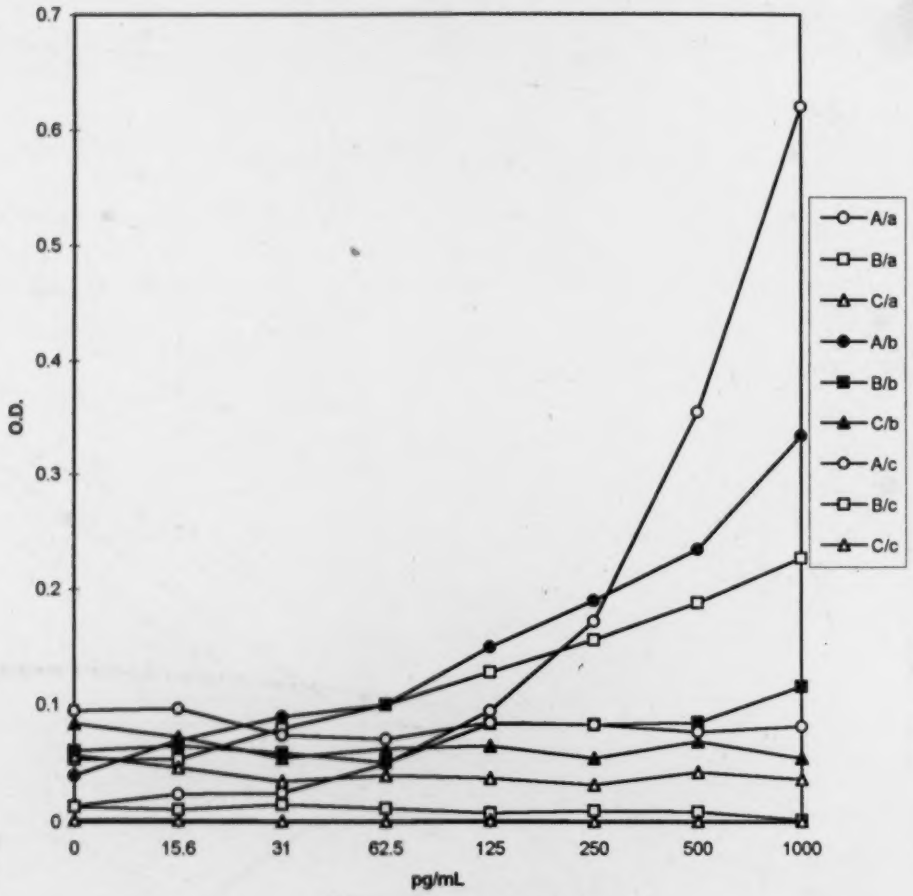


Figure 2-9. IL13 assay - testing three different concentrations of first antibody combined with three different concentrations of second antibody. Legend: letters represent combined concentrations of first antibody (A=1 μg/mL, B=0. 5 μg/mL, C=0.125 μg/mL) and second antibody (a=1 μg/ml, b=0. 5 μg/ml, c=0.125 μg/ml).

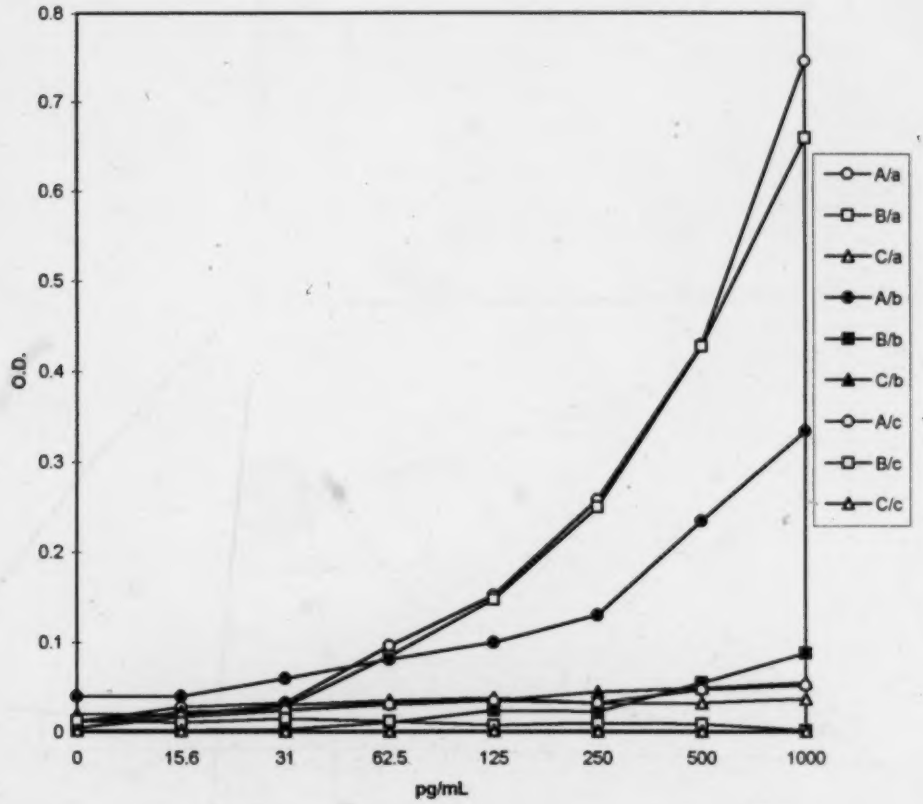


Figure 2-10. TNFα assay - testing three different concentrations of first antibody combined with three different concentrations of second antibody. Legend: letters represent combined concentrations of first antibody (A=1 μg/mL, B=0. 5 μg/mL, C=0.125 μg/mL) and second antibody (a=1 μg/ml, b=0. 5 μg/ml, c=0.125 μg/ml).

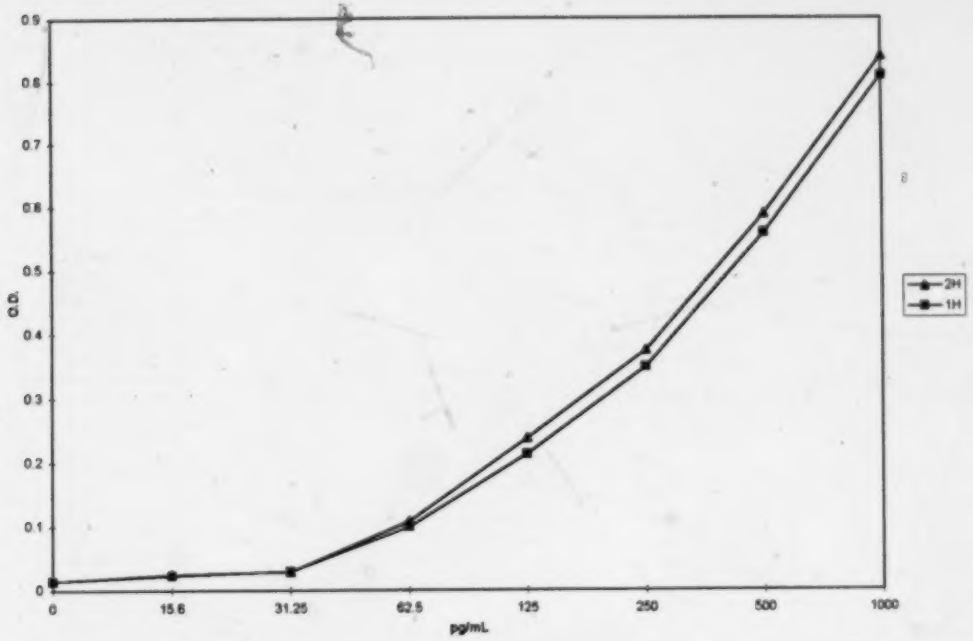


Figure 2-11. IFNγ assay - testing two different incubation periods, one (1H) and two (2H) hours.

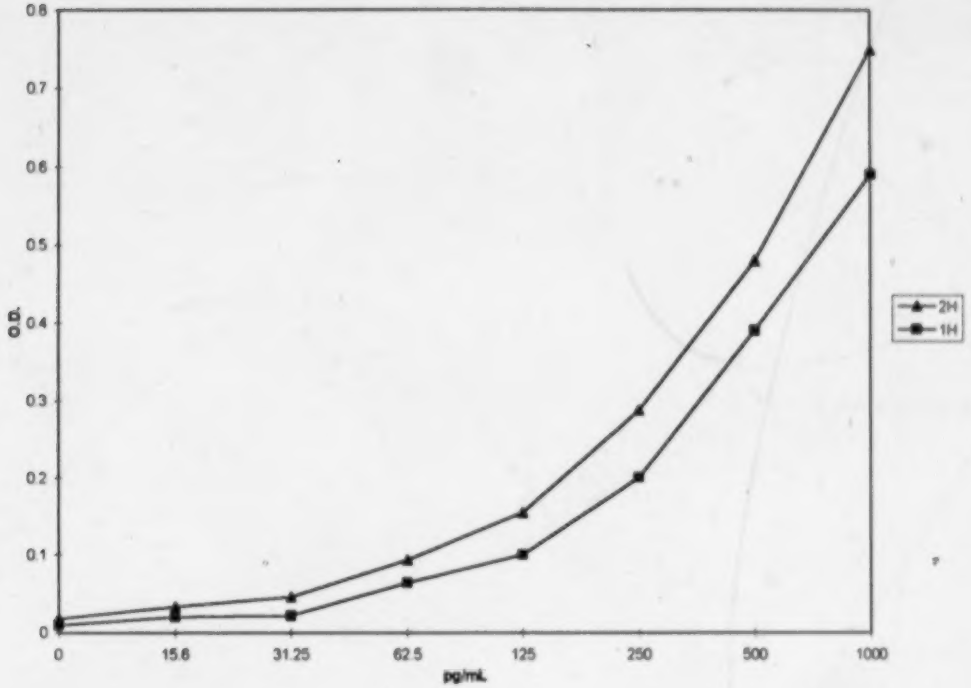


Figure 2-12. IL4 assay - testing two different incubation periods, one (1H) and two (2H) hours.

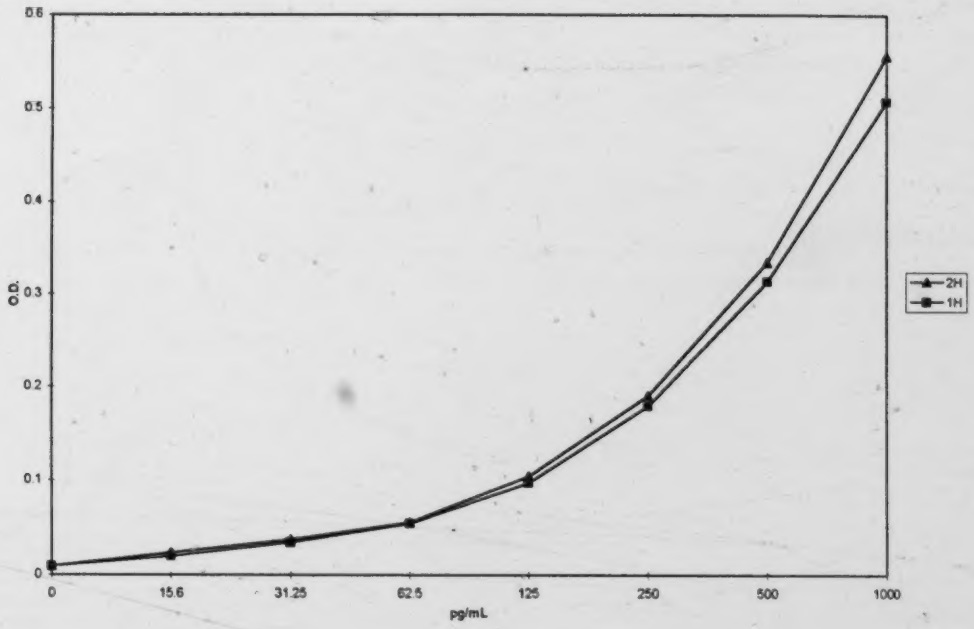


Figure 2-13. IL5 assay - testing two different incubation periods, one (1H) and two (2H) hours.

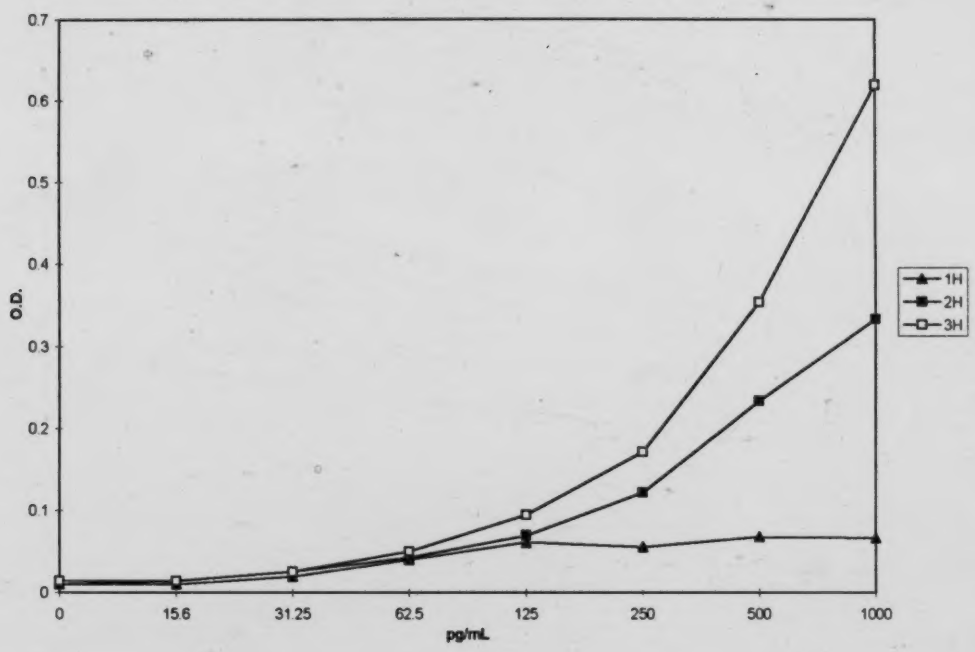


Figure 2-14. IL13 assay - testing three different incubation periods, one (1H), two (2H) and three (3H) hours.

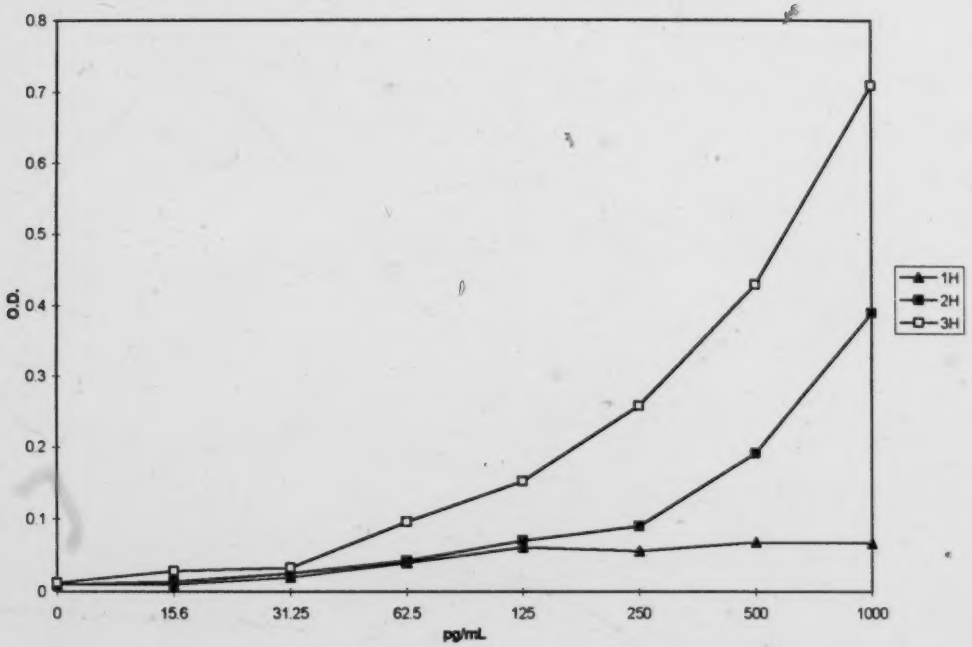


Figure 2-15. TNFα assay - testing three different incubation periods, one (1H), two (2H) and three (3H) hours.

2.4. IMMUNOPHENOTYPING

2.4.1. MONOCLONAL ANTIBODIES:

- anti-CD14 (Leu-3M)PE (phycoerythrin conjugated) 8 µl/test, Becton-Dickinson, San Jose, CA
- matching isotype control (IgG2b) 8 µl/test, Becton-Dickinson, San Jose, CA
- premixed solution of anti-CD3TRICOLOR®/anti-CD4PE/anti-CD8FITC (Fluorescein isothiocyanate conjugated), 8 µl/test, Caltag Laboratories, San Francisco, CA
- premixed solution of matching isotype controls (all IgG2a), Caltag Laboratories, San Francisco, CA.

2.4.2. STAINING PROCEDURE

a) PBMC (5×10^5), diluted with 100 µl cold buffer (PBS with 0.1% NaN_3), were placed in 5 mL tube.

b) appropriate volume of MAb or isotype control was added to the tube and mixed.

c) after 30 minutes of incubation at 4°C, 3 mL of cold buffer were added and tubes were centrifuged for 5 minutes (2000 rpm). Buffer was decanted and drained on absorbent paper.

d) cells were resuspended in 0.4 mL of cold 1% formalin and kept at 4 °C until analysis.

2.4.3. FLOW CYTOMETRY

Stained cells were analyzed on a FACScan flow cytometer equipped with an argon laser (excitation wavelength 488 nm; Becton-Dickinson) using Cell Quest™ software. An electronic gate was set around the lymphocyte clusters visible on a two-parameter dot plot of 90^0 (side scatter) versus forward angle scatter to measure proportion of T cells. The gate for lymphocytes was widely set to include small as well as large lymphocytes and to avoid bias towards a particular subpopulation. The gate was kept constant for each experiment. Twenty thousand gated events were recorded for the premixed solution of anti-CD3TRI-COLOR®/anti-CD4PE/anti-CD8FITC marker and control. Two-color dot plots were used (FL3vs.FL1 and FL3vsFL2 where FL3 describes $CD3^+$ cells) to determine the percentage of CD4 and CD8 positive T cells. A marker was set on the appropriate control plot such that 1% or less of the cells were to the right of this channel marker. Combination of anti-CD3 and anti-CD8 antibodies was useful to distinguish $CD8^+$bright T-cells from NK lymphocytes which are $CD3^-$ $CD8^+$ dim.

Anti-CD14 was used to measure the amount of monocytes in the sample. Dead cells, erythrocytes and platelets were excluded from analysis by setting appropriate threshold trigger on low forward and side scatter parameters. Percentage of $CD14^+$ cells was calculated using FL2vsFSC dot plot after a cut-off for positive events was set on appropriate control panel. The population of $CD14^+$ cells was distinct from the lymphocyte population. Five thousand gated events were recorded for this marker and its control.

3. RESULTS

3.1. MANTOUX RESULTS

The subjects were divided in three groups according to their Mantoux status (shown in figure 3-1). Group 1 were the positive Mantoux, group 2 were the negative Mantoux with tuberculosis contact, and group three were the negative Mantoux subjects with no tuberculosis contact.

3.2. PPD RESPONSE

3.2.1 IFNγ PRODUCTION

There were some individuals who had high spontaneous release of IFNγ and four of them had offscale values at some point in time (shown by stars in figure 3-2). Since all IFNγ results were subtracted from control cultures, results for the days with offscale controls were not shown (unless otherwise specified). There were some offscale values for the PPD stimulated cultures that could not be repeated for lack of sample (they are plotted at the 1000 pg/mL level).

Some individuals were highly responsive to even the lower dose of PPD (figure 3-3). Different individuals showed different responses to stimulation with PPD. IFNγ production was higher on days four and six of culture in a dose dependent manner (figures 3-3, 3-4, 3-5). All subjects had IFNγ production with the higher dose of PPD (figure 3-5).

The correlation of IFNγ production before and after Mantoux is shown for the different days and for each PPD dose in figures 3-6, 3-7 and 3-8. The curve slopes were shown for correlations with r^2 greater than 0.4. The correlation was greater at higher PPD concentrations. At lower levels of PPD the highest r^2 seen is 0.454 whilst at the larger dose of PPD the highest r^2 is 0.771. It is also clear that the degree of correlation is dependent on the culture day tested. Seen in

figure 3-8, at the higher dose of PPD day 4 of culture showed the best correlations, dropping at day 6 even though this day had the higher amounts of IFNγ production. In figure 3-9, when adding the offscale values (vertical arrows denote offscale values after Mantoux and horizontal arrow denotes offscale values before Mantoux) it can be seen that high values of IFNγ production were independent of Mantoux status. The figure also shows that no clear pattern is observed for the three different groups and, for the individuals that had a change of IFNγ production after Mantoux, there was no bias towards a higher or a lower response before or after Mantoux. However, some persons had a higher spontaneous release of IFNγ after Mantoux (figure 3-10) but there was no statistical difference. The standard deviations for the triplicates of IFNγ production upon stimulation with 50 μg/mL of PPD and control cultures before and after Mantoux, for the four days of culture, are shown in figure 3-26.

3.2.2. THYMIDINE INCORPORATION (TI)

Figure 3-11 shows that almost all subjects, with exception of one, had spontaneous TI lower than 1000 counts per minute (cpm). The TI response to PPD increased, for most subjects, with the higher dose of PPD as shown in figure 3-12. The same figure shows that there is a large response variation between different individuals but there was no statistical difference between the different groups. However, group three showed a group of individuals (represented in the right bottom at figure 3-12) with apparently higher PPD-induced TI responses for all the concentrations tested when compared to the other individuals within that group.

Importantly, there was a consistency in the TI response to PPD for the same individual. As seen in figure 3-13, the correlation of the TI response before and after Mantoux showed a r^2 of 0.746 for the intermediate dose of PPD (10 μg/mL) and a r^2 of 0.659 for the larger dose (50 μg/ml).

3.2.3. CORRELATIONS BETWEEN TI AND IFNγ RESPONSE

The TI response to the lower dose of PPD (2 μg/mL) did not show a significant correlation with the IFNγ response except for the first day of culture when the values for IFNγ production were, for most subjects, extremely low (figure 3-14). The TI response to the intermediate dose of PPD (10 μg/mL) showed the best correlation with PPD-stimulated IFNγ production which was detected at day four with an r^2 of 0.516 and at day six with a r^2 of 0.827 (figure 3-15). Even when the data are separated into before and after Mantoux results, the correlations remained practically the same (figure 3-16). The TI response to the larger dose of PPD (50 μg/mL) also had a good correlation with PPD-stimulated IFNγ production on all days tested but day four was the best (figure 3-17).

3.2.4. OTHER CYTOKINES

There was no production of IL4 upon stimulation with the three concentrations of PPD with exception of one individual who produced IL4, before the Mantoux test, on the first day supernatants from cultures stimulated with 10 and 50 μg/mL of PPD (Appendix). IL5 was tested in 13 subjects. It was produced by a few of them, mostly at day four and mostly with the higher PPD dose (figure 3-18). IL13 was tested in 8 subjects and it was not produced at all with the lower dose of PPD. The higher PPD dose had a few weak responders mostly at days four and six ((figure 3-19). TNFα production was tested in 8 subjects and was detected in all three PPD concentrations in a dose dependent manner. The kinetics of detection were different from the other cytokines since there was an early peak at day one with subsequent decline up to day four and another peak at day six that was most evident with the larger PPD dose (figure 3-20).

3.3. CANDIDA AND TETANUS RESPONSE

3.3.1. IFNγ PRODUCTION

Most subjects had a weak IFNγ response to tetanus and, for the subjects with a higher response, it was more evident at day four of culture (figure 3-21). The candida response was also weak and most expressed at day six of culture (figure 3-22). Most subjects had no significant difference in IFNγ production after the Mantoux for both candida and tetanus response.

3.3.2. THYMIDINE INCORPORATION

Half of the subjects had a good response to tetanus (more than 1000 cpm) in contrast to the candida response that was mostly weak (Appendix). There was no statistical difference before and after Mantoux.

3.3.3. OTHER CYTOKINES

There was no detectable IL4 production for either candida and tetanus antigens (Appendix). IL5 response to candida was detected in just two subjects (Appendix). Upon tetanus stimulation, a few subjects produced IL5, mostly at days four and six (Appendix). IL13 response to candida and tetanus was detected in just a few subjects and mostly at later days of culture (Appendix). Most subjects produced TNFα in response to both antigens early in culture, dropping after day one but still detectable at later days(Appendix).

3.4. PHA RESPONSE

3.4.1 IFNγ RESPONSE

Since we were more interested in the specific antigenic response to PPD, the results for PHA are not discussed in detail. All subjects had a high response to PHA in both concentrations tested (2 and 10 μg/mL) and some values were offscale (Appendix). The IFNγ production upon the higher concentration of PHA showed a strong correlation before and after Mantoux test (figure 3-23).

3.4.2 THYMIDINE INCORPORATION

The TI response to PHA was very similar before and after Mantoux (Appendix).

3.4.3 OTHER CYTOKINES

IL5, IL13 and TNFα were produced in higher amounts in both PHA concentrations tested and many results were offscale (Appendix). IL4 was also detected after stimulation with both PHA concentrations at days two and four and also at day six for the higher concentration of PHA (Appendix).

3.5. CONTROL CULTURES

Spontaneous release of IFNγ and spontaneous TI results were already presented at the PPD response section. A few subjects had spontaneous release of IL5. The same few subjects also had detectable IL13 (Appendix). Most of the subjects tested had detectable spontaneous release of TNFα at the first day of culture (Appendix).

3.6. IMMUNOPHENOTYPING RESULTS

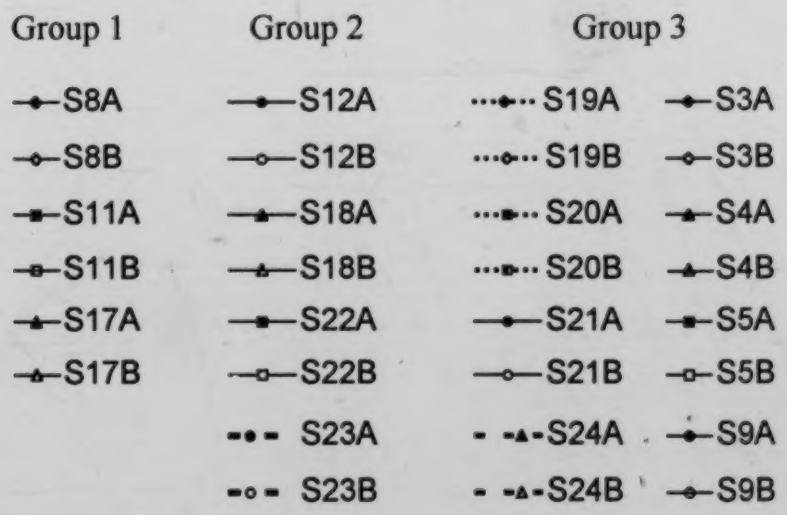
The PBMC samples showed no statistical difference in the amounts of CD4 and CD8 positive cells before and after Mantoux testing (figure 3-24).

There was also no clear pattern between the three different groups. The amount of CD14 positive cells (macrophages) was mostly higher in groups one and two. Also, in most cases in these two groups there was an increase in CD14 positive cells after Mantoux (figure 3-25).

	SUBJECTS	MANTOUX SIZE (mm)
GROUP 1 (Mantoux positive)	S8	18x18
	S11	18x18
	S17	9x9
GROUP 2 (Mantoux negative, contact with tuberculosis)	S12	0x0
	S18	0X0
	S22	0x0
	S23	0x0
GROUP 3 (Mantoux negative, no contact with tuberculosis)	S3	0x0
	S4	0x0
	S5	0x0
	S9	0x0
	S19	0x0
	S20	0x0
	S21	0x0
	S24	0x0

Figure 3-1. Mantoux results from 15 subjects divided into three groups.

General legend for figures 3-2 to 3-22 unless otherwise specified is presented below. Code A identifies samples taken before Mantoux testing and code B for samples taken after Mantoux testing. Results offscale for IFNγ are noted by star (*).



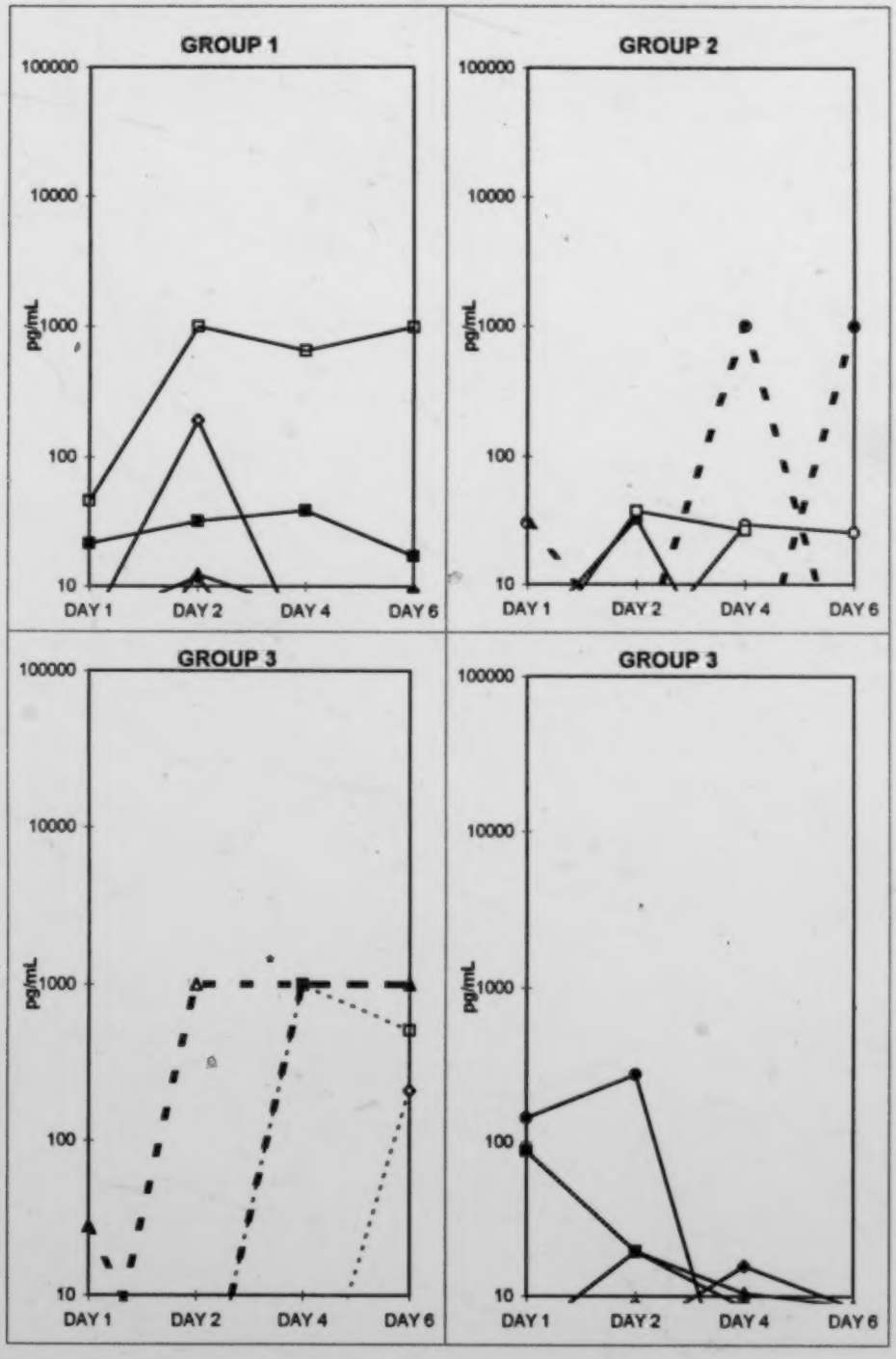


Figure 3.2. IFNγ production by PBMC without antigenic stimulation (control cultures).

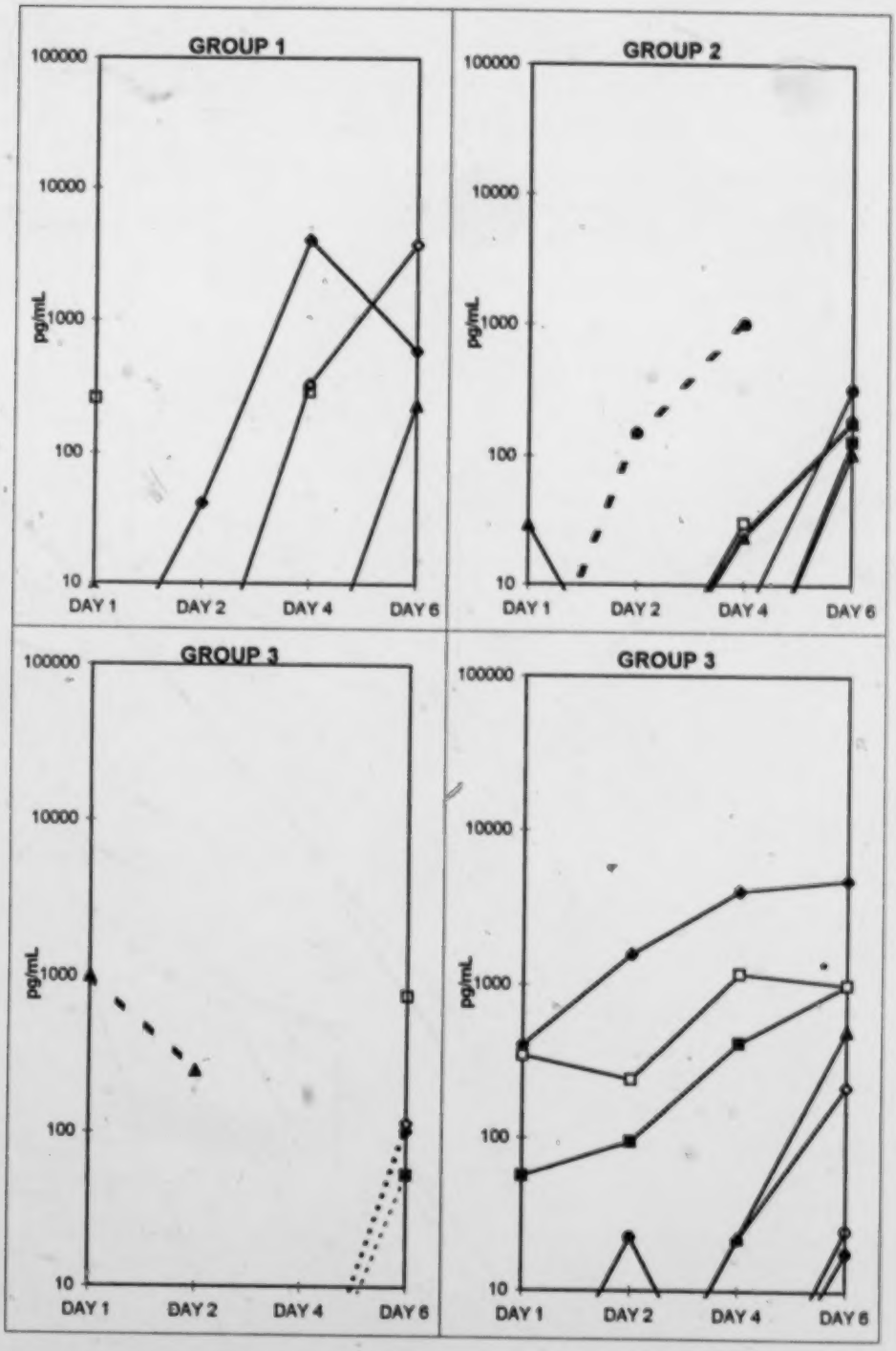


Figure 3.3. IFNγ production by PBMC upon stimulation with 2 ug/mL of PPD.

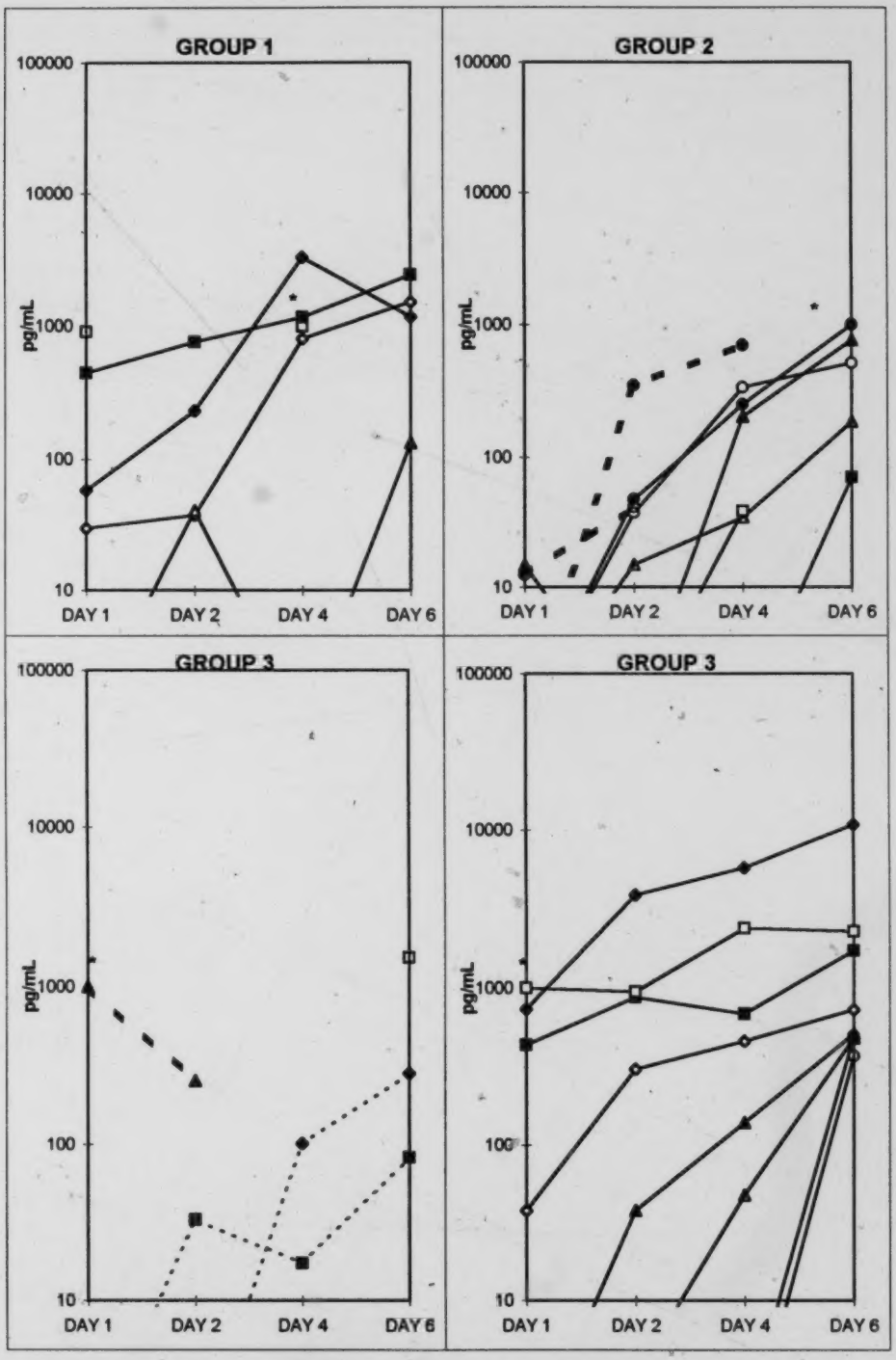


Figure 3.4. IFNγ production by PBMC upon stimulation with 10 ug/mL of PPD.

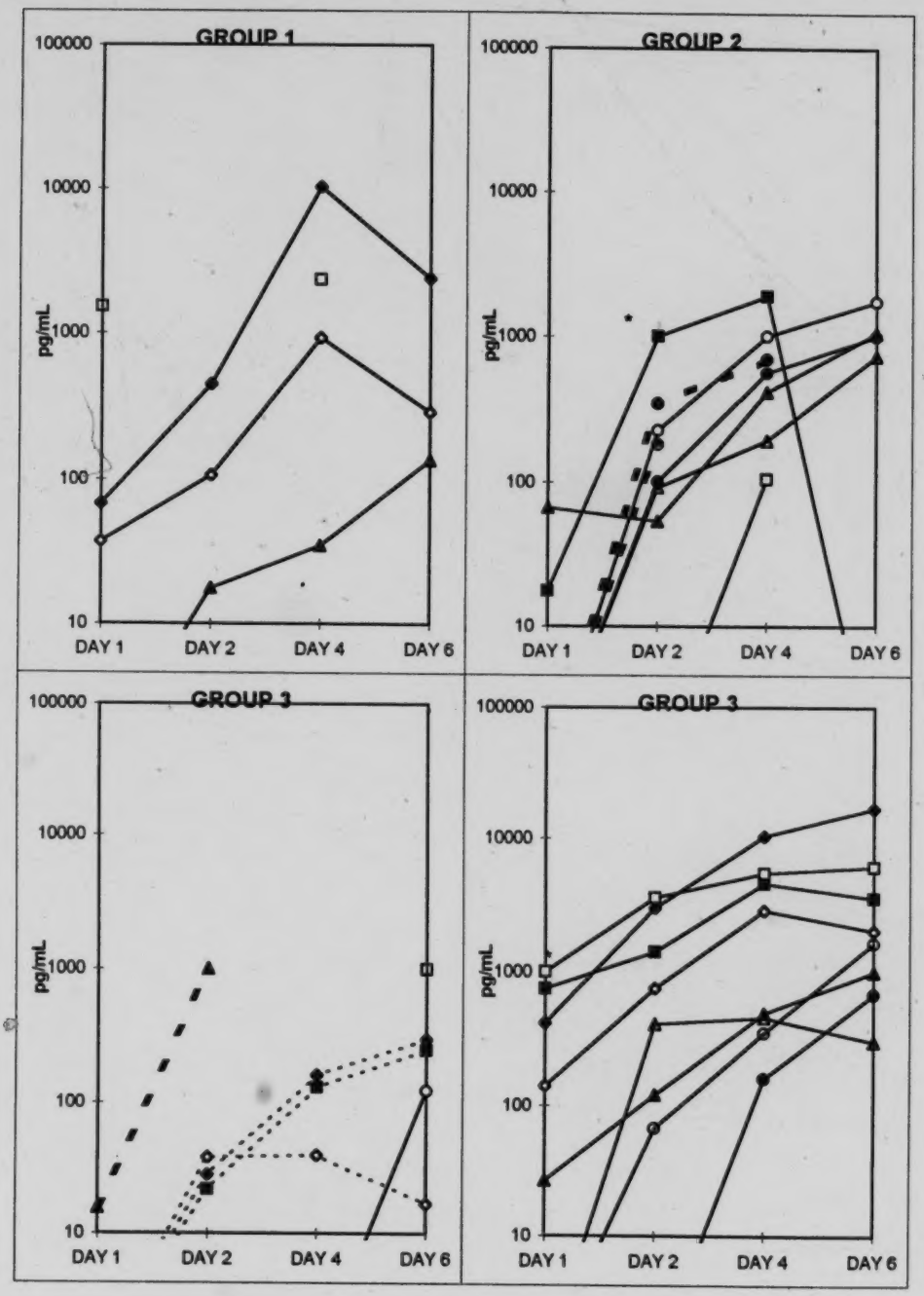


Figure 3-5. IFNγ production by PBMC upon stimulation with PPD 50 ug/mL

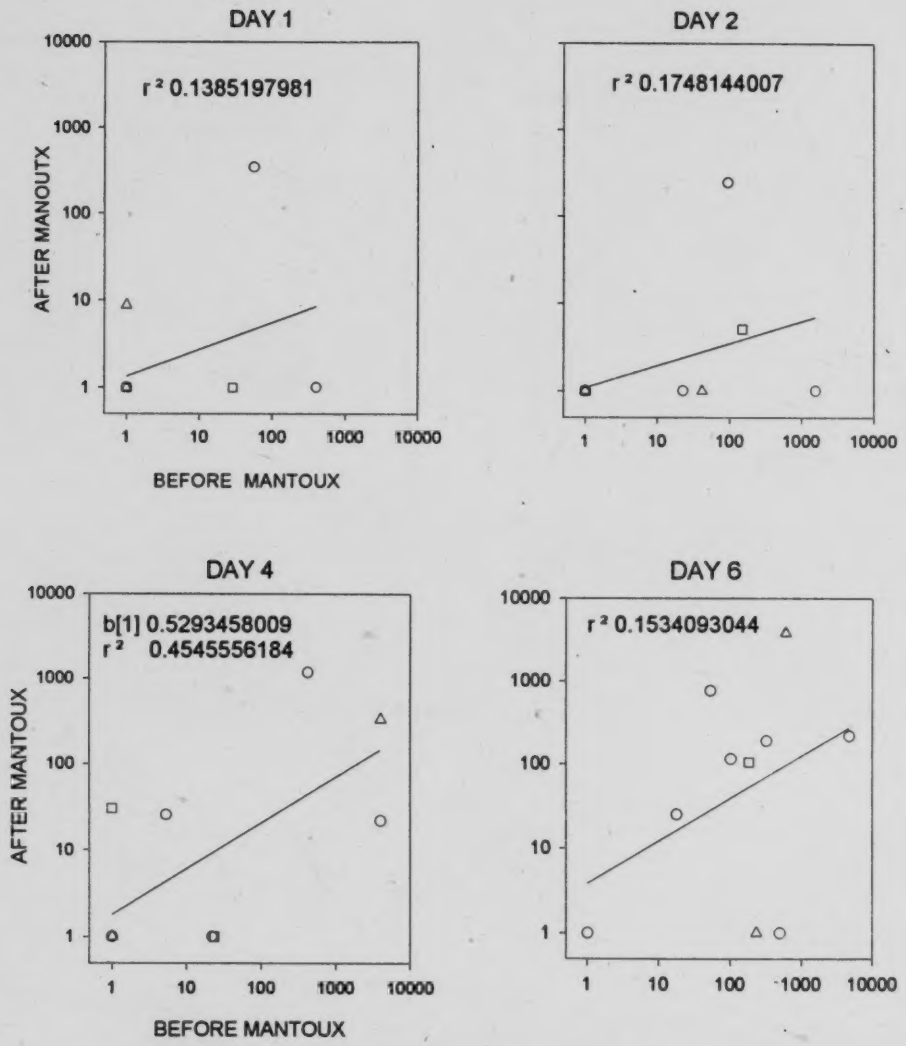


Figure 3-6. IFNγ production by PBMC upon stimulation with 2 ug/mL of PPD before and after Mantoux. Triangles are individuals from group1, square from group 2, and circles from group 3.

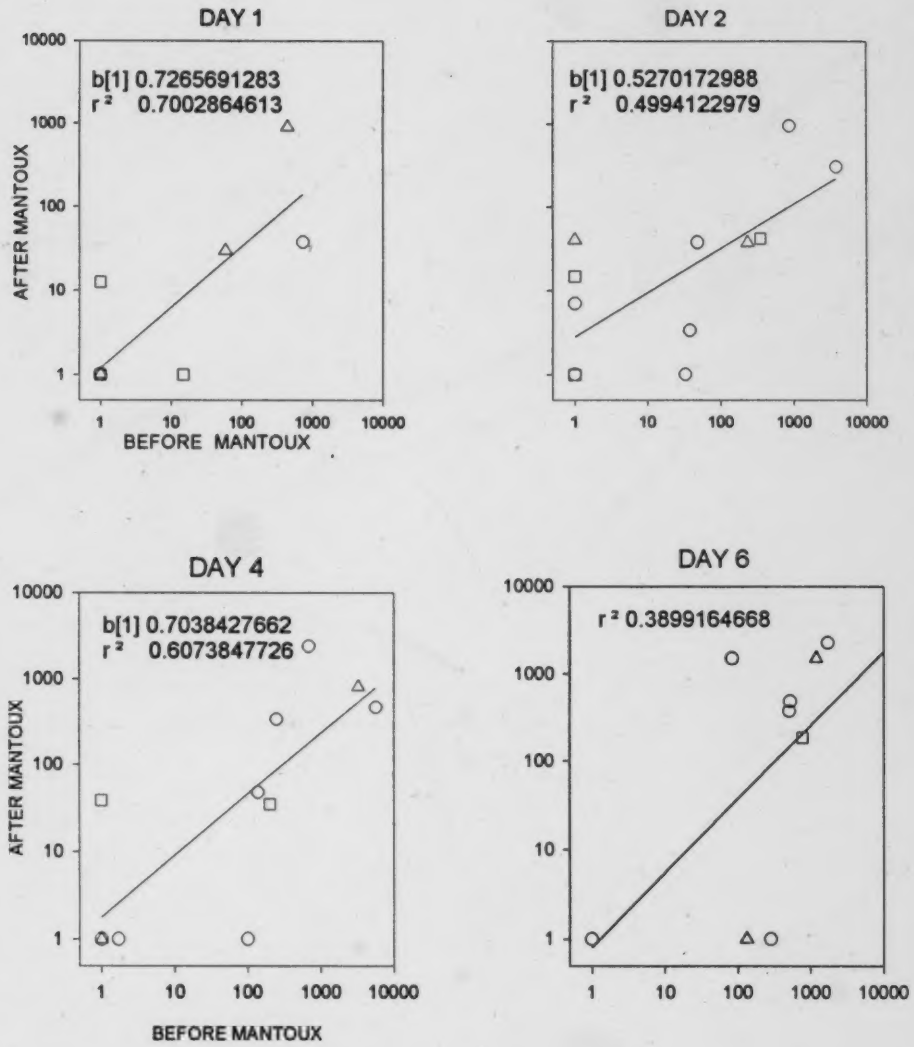


Figure 3-7. IFNγ production by PBMC upon stimulation with 10 ug/mL of PPD before and after Mantoux. Triangles are individuals from group1, square from group 2, and circles from group 3.

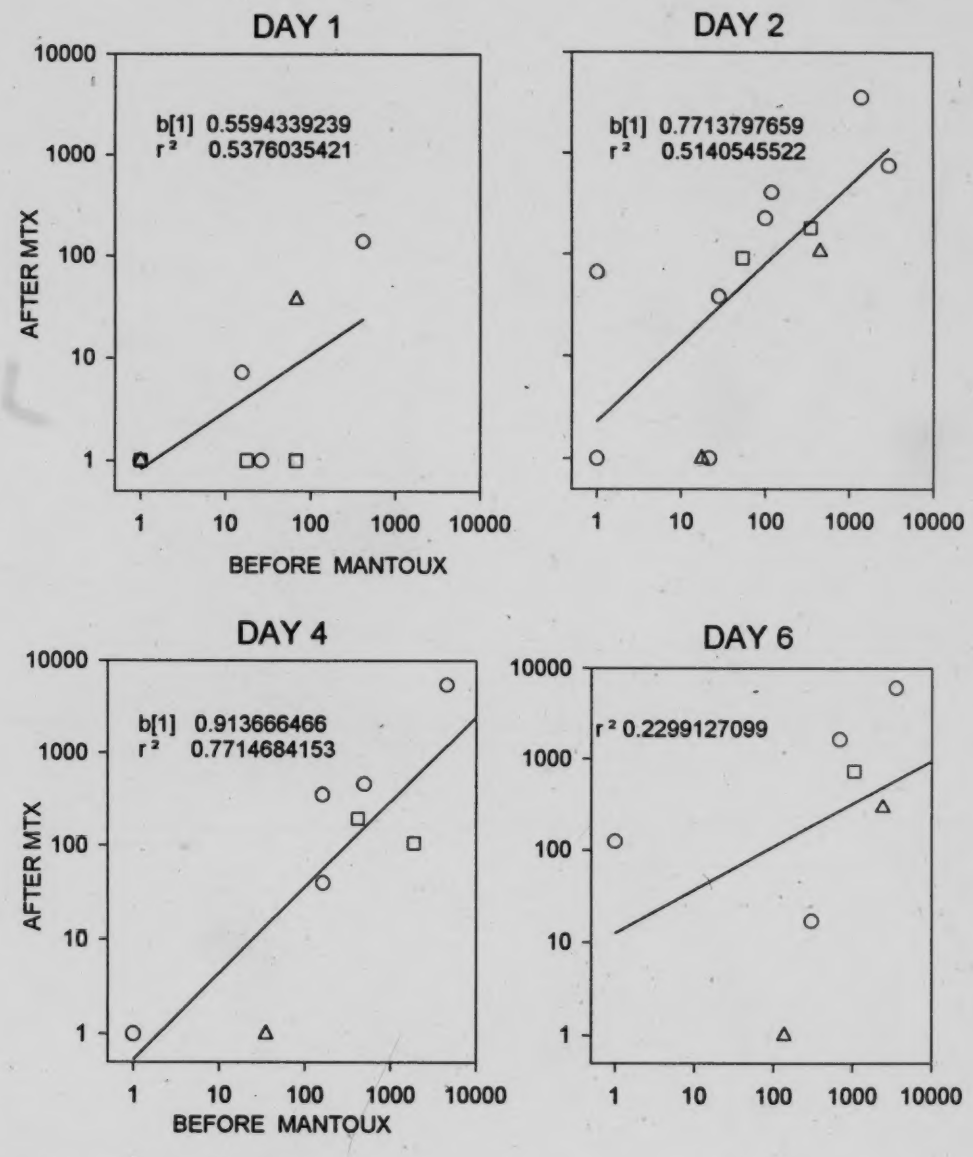


Figure 3-8. IFNγ production by PBMC upon stimulation with 50 ug/mL of PPD before and after Mantoux. Triangles are individuals from group1, square from group 2, and circles from group 3.

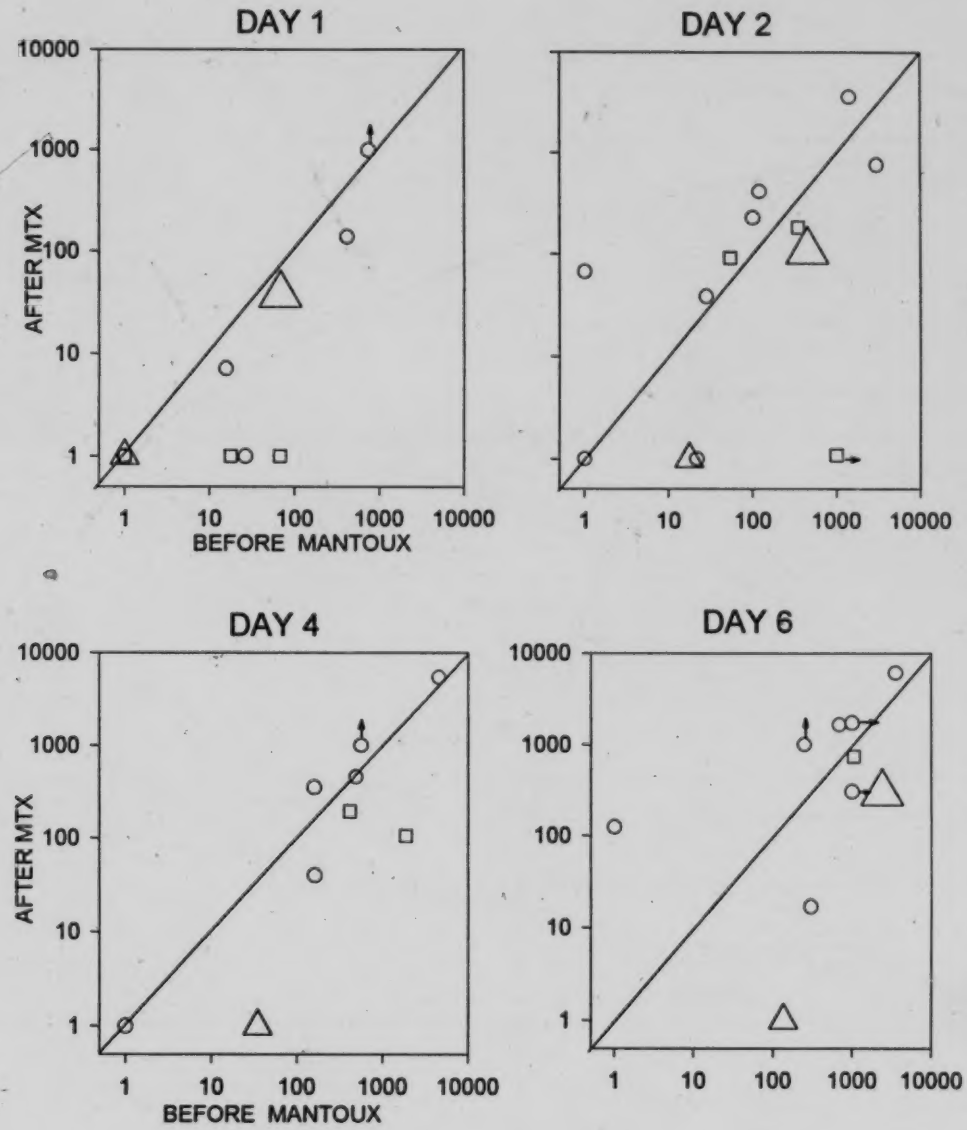


Figure 3-9. IFNγ production (in pg/mL) by PBMC upon stimulation with 50 ug/mL of PPD before and after Mantoux. Arrows indicate offscale values. Size of symbols correspond to size of Mantoux reaction (smaller symbols correspond to a 0x0 mm, intermediate to a 9x9 mm,and larger to a 18x18 mm skin reaction). Triangles are individuals from group1, squares from group 2, and circles from group 3. The diagonal line is for visual purposes.

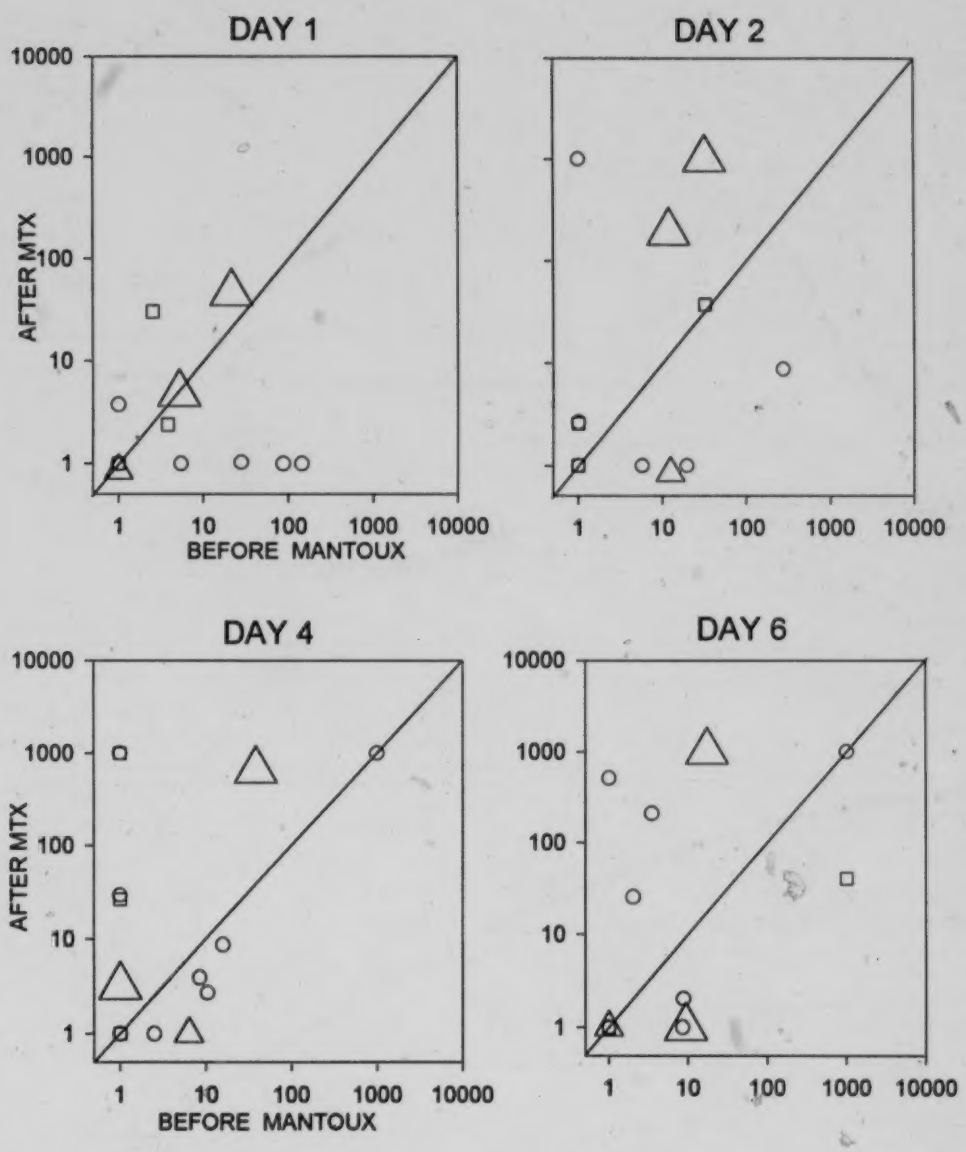


Figure 3-10. IFNγ production (in pg/mL) without antigenic stimulation (control cultures). Size of symbols correspond to size of Mantoux reaction (smaller symbol correspond to a 0x0 mm, intermediate to a 9x9 mm,and larger to a 18x18 mm skin reaction).Triangles are individuals from group1, square from group 2, and circles from group 3.

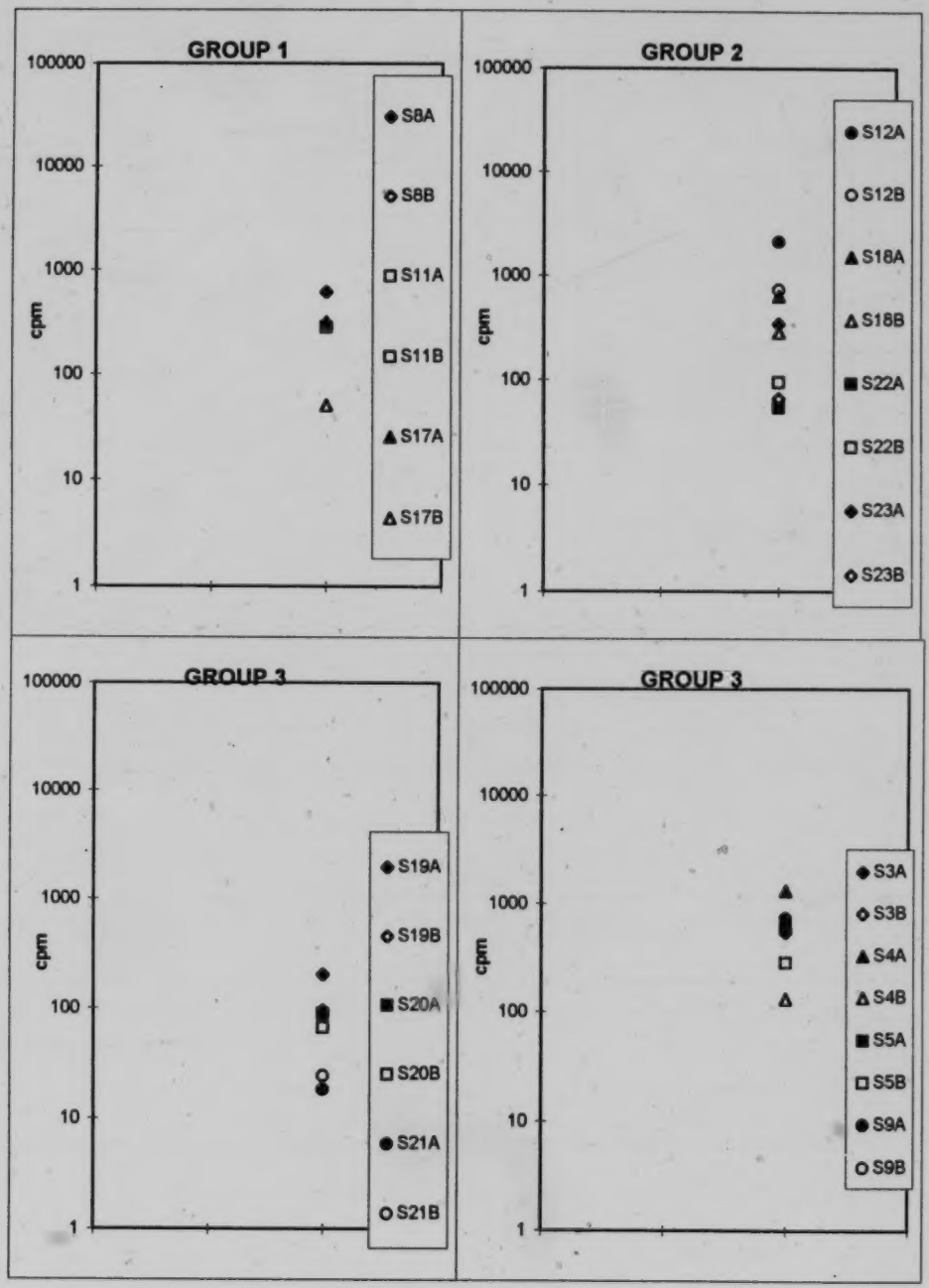


Figure 3-11. TI by PBMC without antigenic stimulation (control cultures). Results are in counts per minute (cpm).

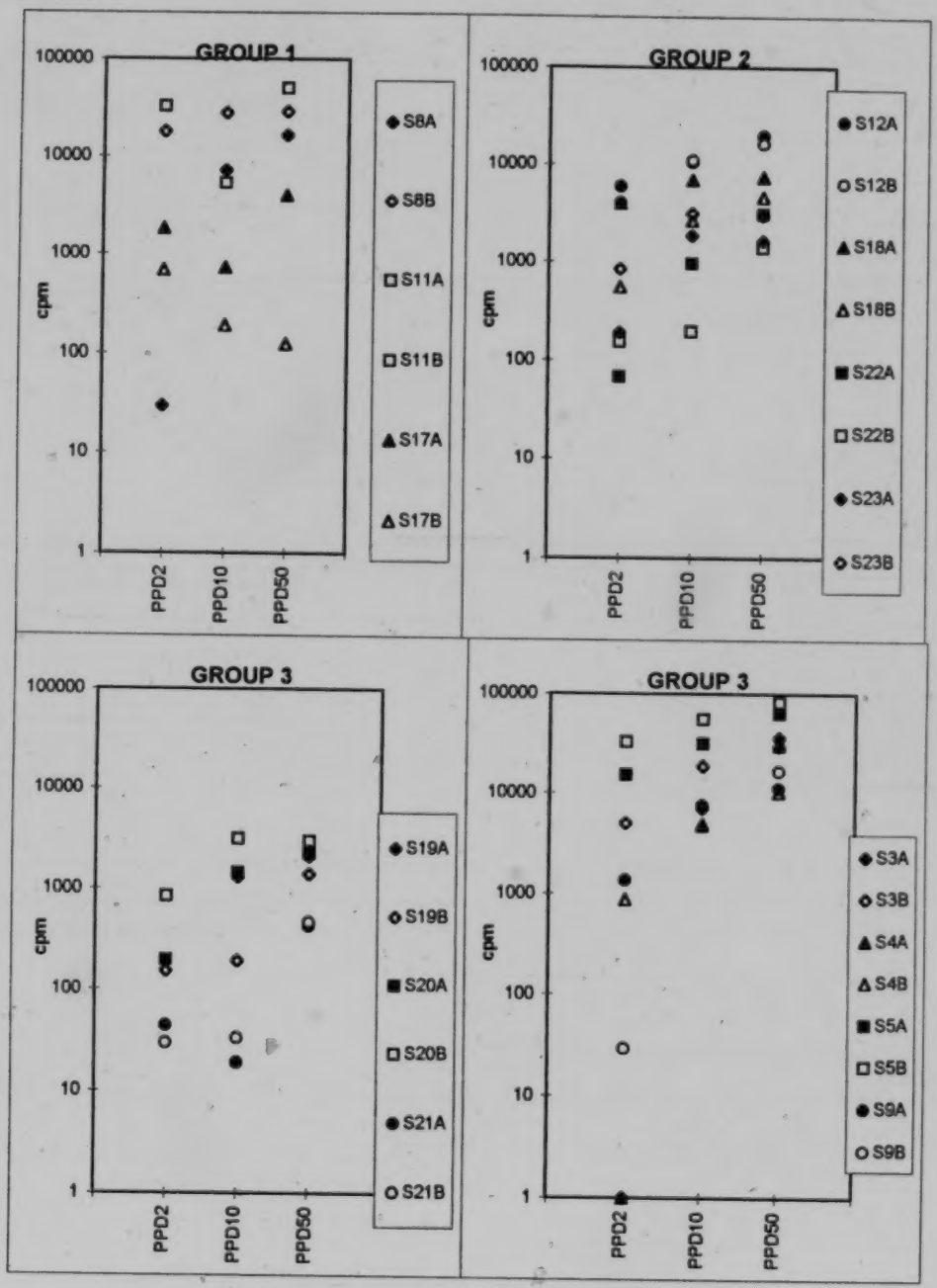


Figure 3-12. TI by PBMC upon stimulation with three different concentrations of PPD (2, 10 and 50 ug/mL). Results are in counts per minute (cpm).

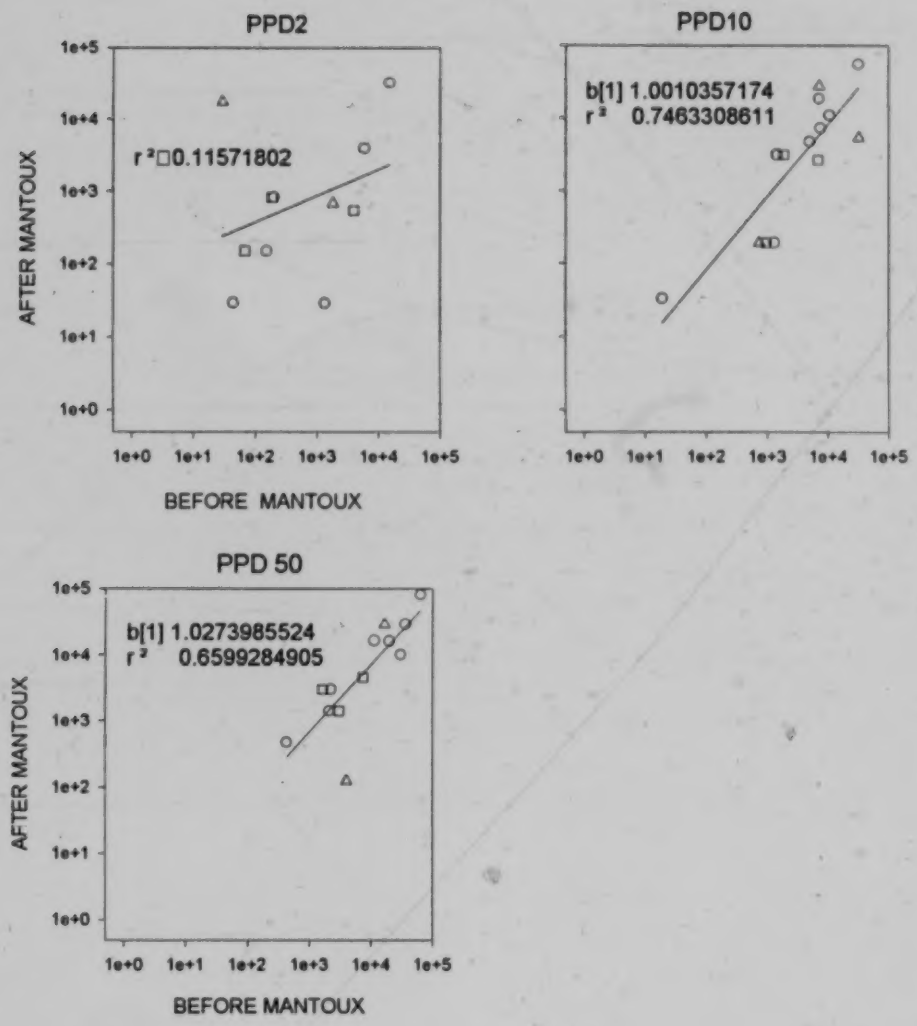


Figure 3-13. BT upon stimulation with three different doses of PPD before and after Mantoux (PPD 2= 2 ug/mL; PPD 10= 10 ug/mL; PPD 50= 50 ug/mL).
Triangles are individuals from group1, square from group 2, and circles from group 3.

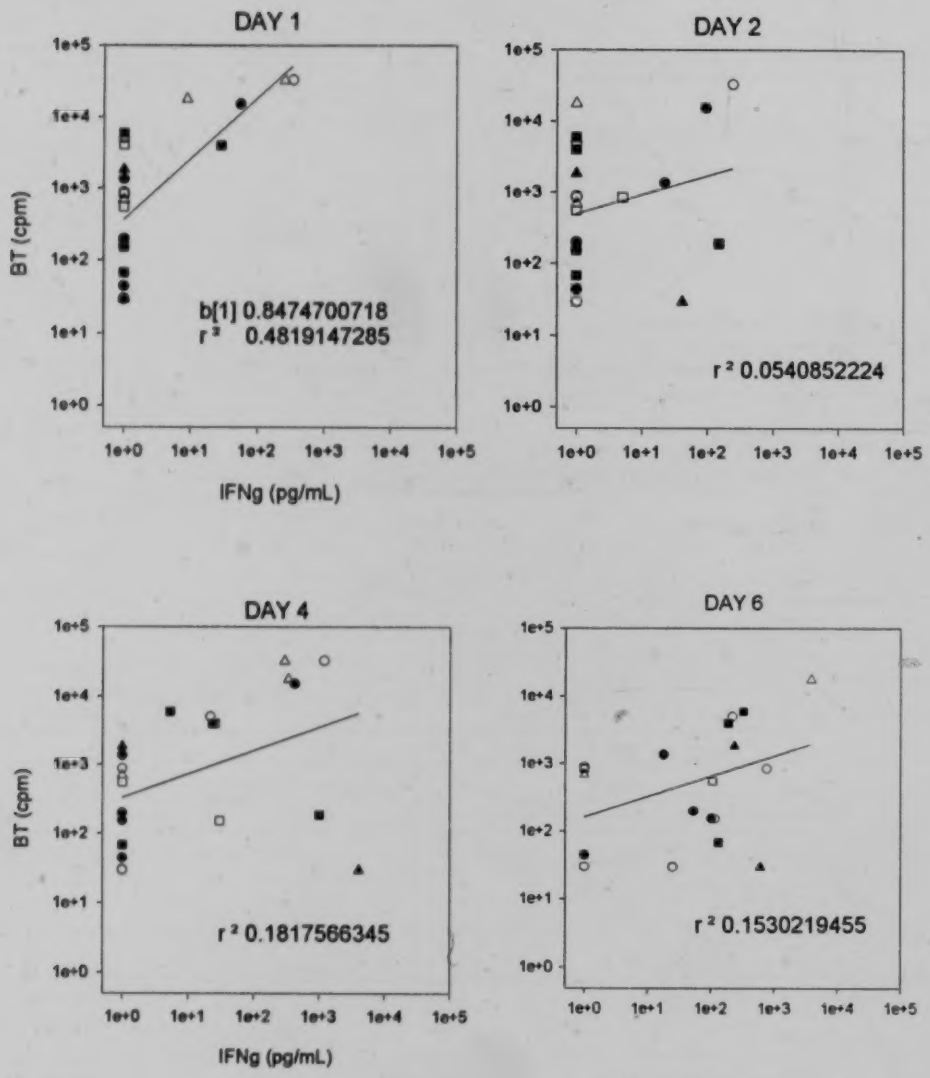


Figure 3-14. IFNγ production versus BT upon stimulation with 2 ug/mL of PPD.

Triangles are individuals from group1, square from group 2, and circles from group 3. Solid symbols are A samples and empty symbols are B samples.

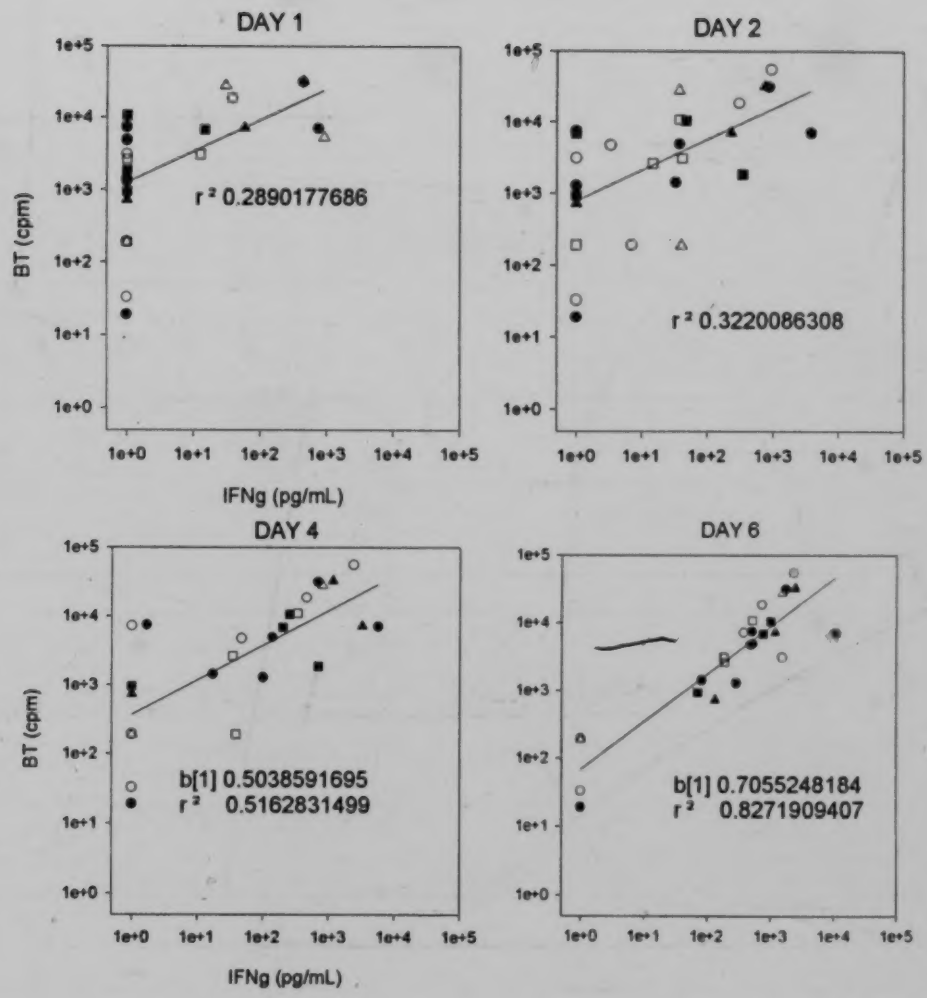


Figure 3-15. IFNγ production versus BT upon stimulation with 10 ug/mL of PPD.
Triangles are individuals from group1, squares from group 2, and circles from group 3. Solid symbols are A samples (before Mantoux test) and empty symbols are B samples (after Mantoux test).

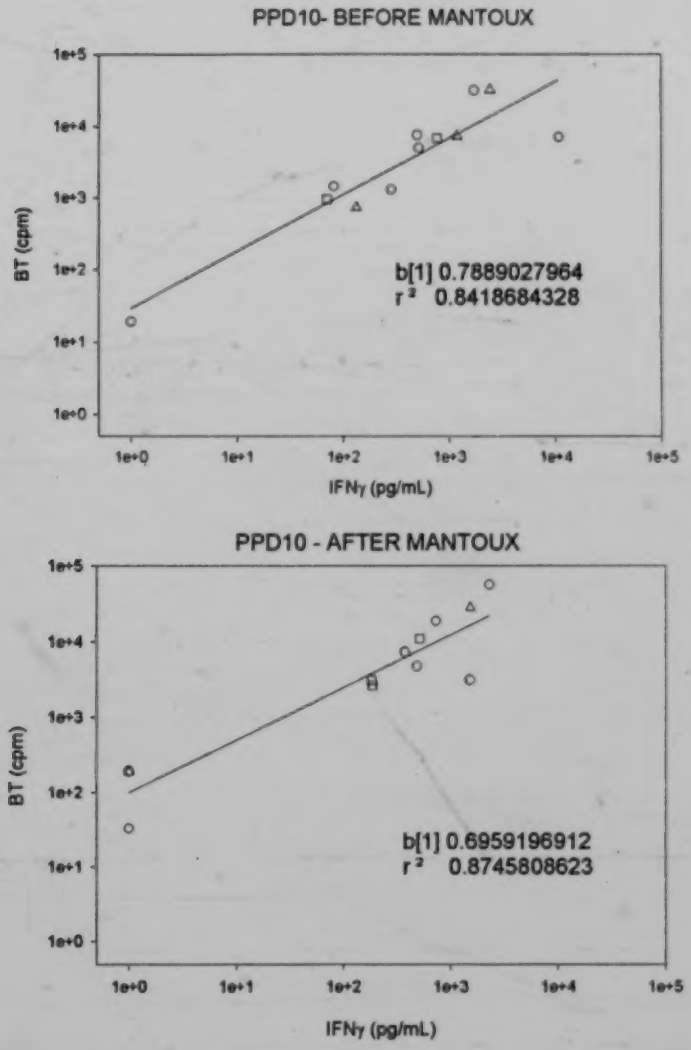


Figure 3-16. IFNγ production versus BT before and after Mantoux test in day six supernatants from cultures stimulated with PPD 10 ug/mL
Triangles are individuals from group1, squares from group 2, and circles from group 3.

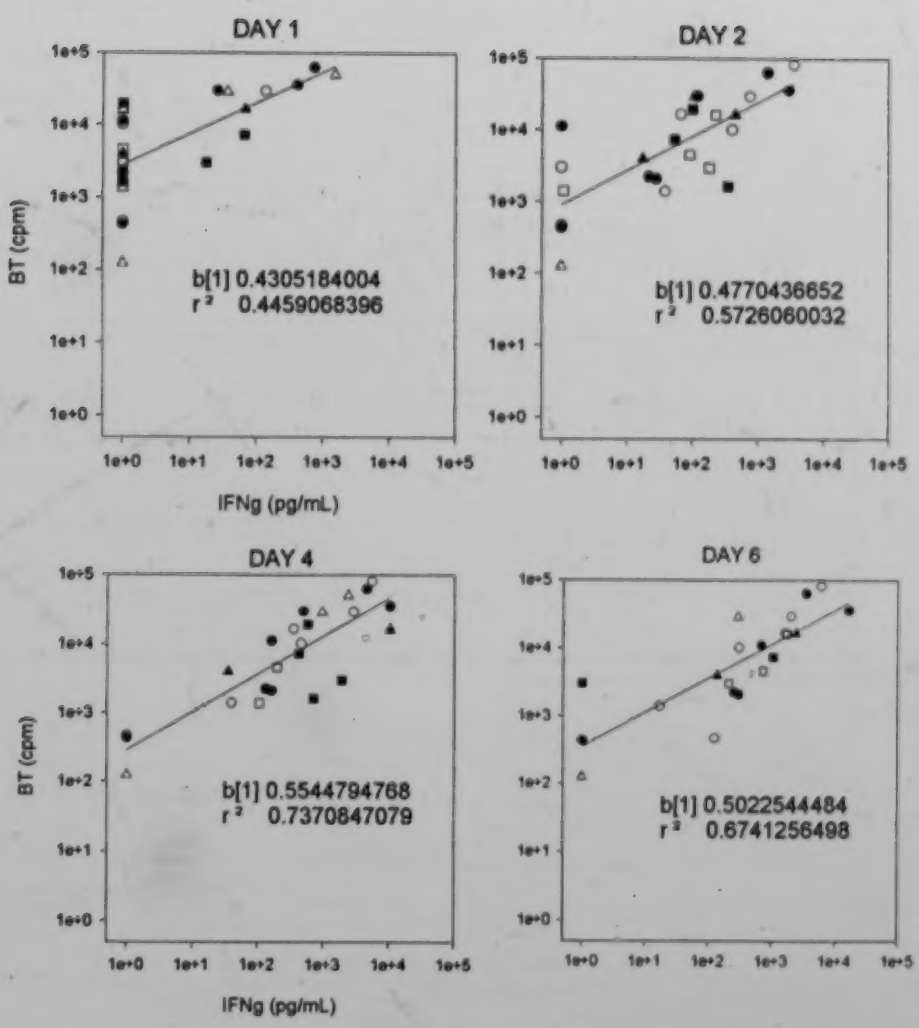


Figure 3-17. IFNγ production versus BT upon stimulation with 50 ug/mL of PPD.

Triangles are individuals from group1, squares from group 2, and circles from group 3. Solid symbols are A samples and empty symbols are B samples.

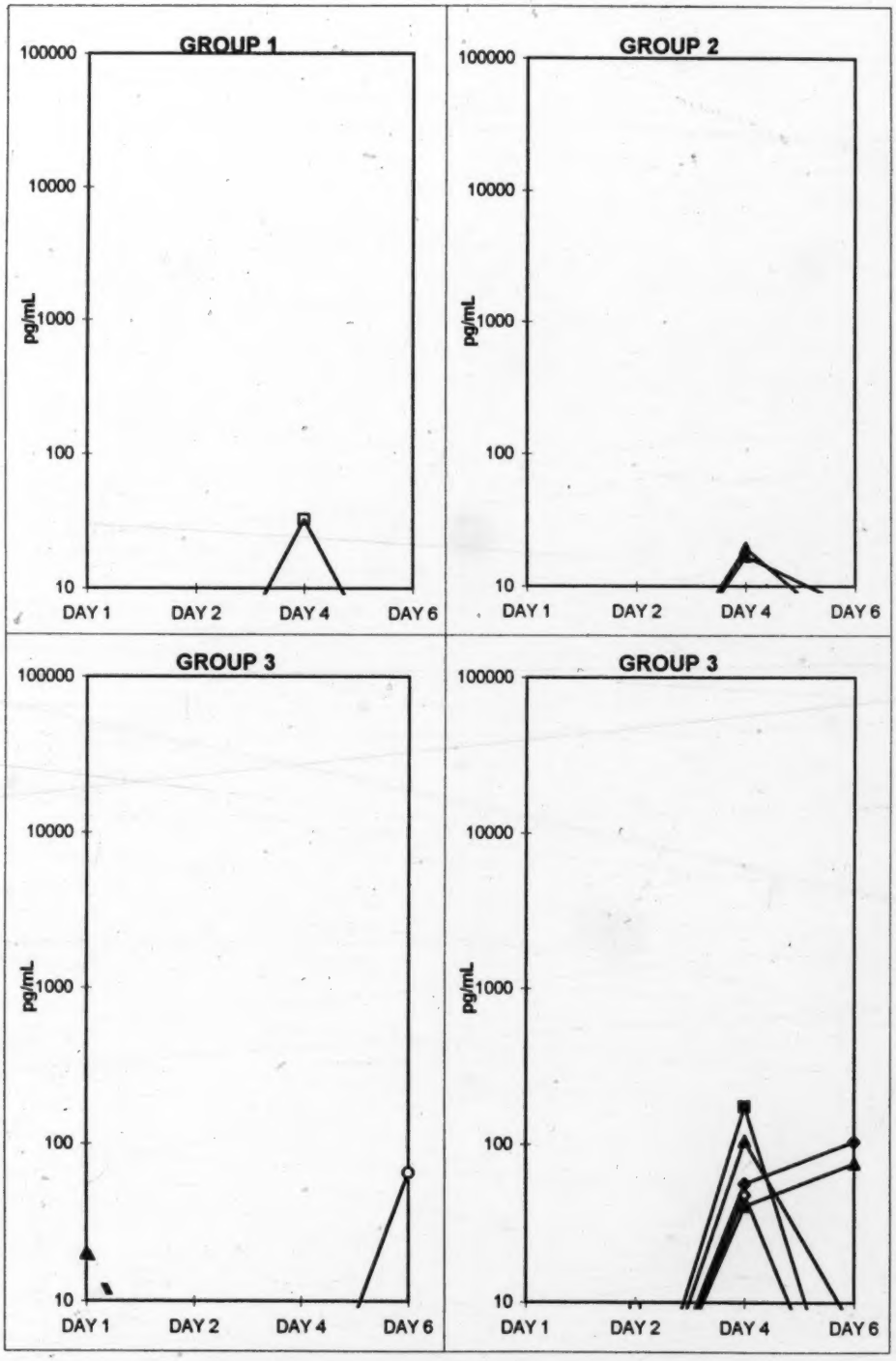


Figure 3-18. IL5 production by PBMC upon stimulation with 50 ug/mL of PPD.

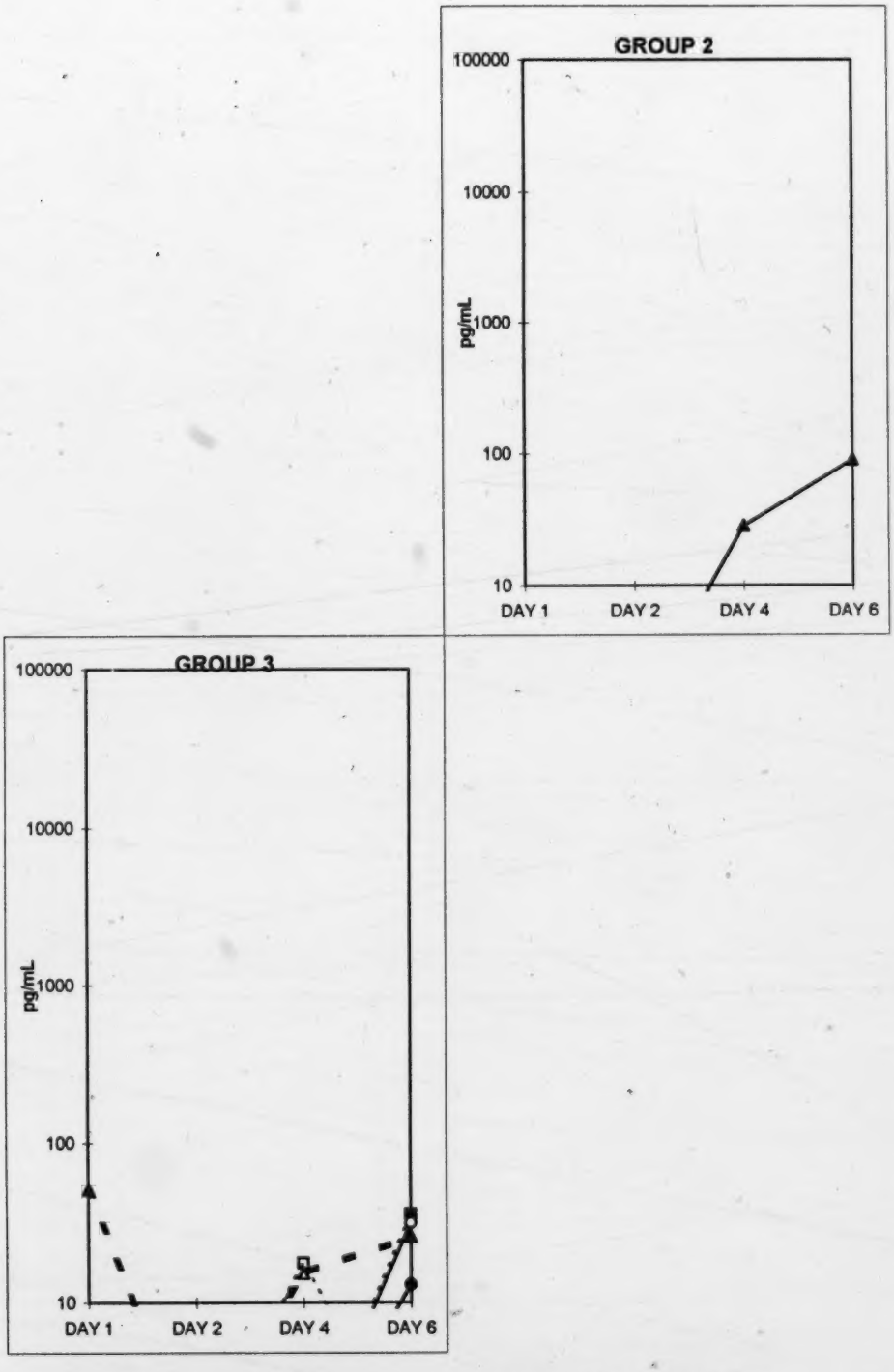


Figure 3-19. IL13 production by PBMC upon stimulation with 50 ug/mL of PPD. Empty spaces correspond to the non tested groups (group 1 and part of group 3).

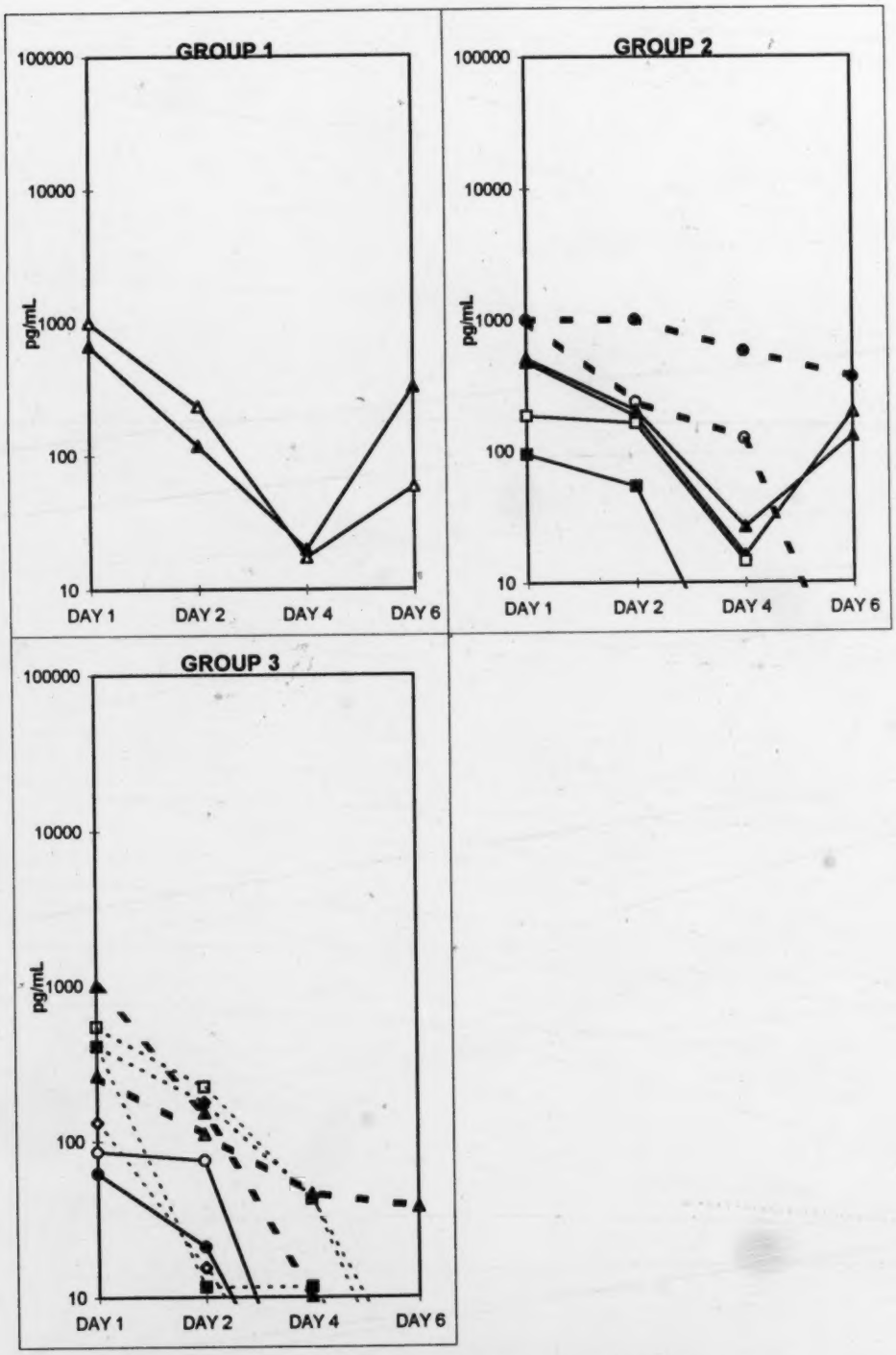


Figure 3-20. TNFα production by PBMC upon stimulation with 50 ug/mL of PPD. Empty space correspond to the non tested group (part of group 3).

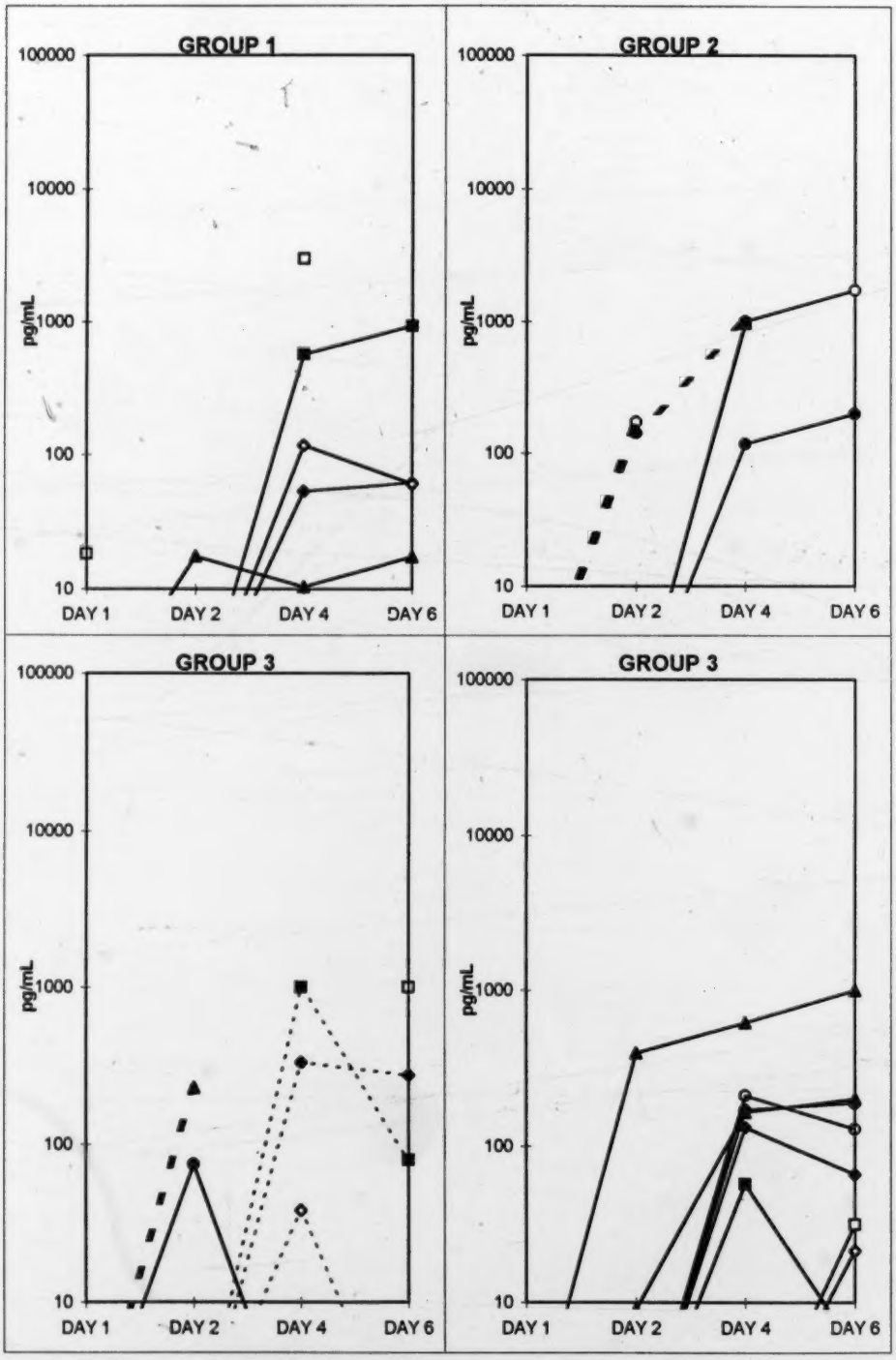


Figure 3-21. IFNγ production by PBMC upon stimulation with tetanus.

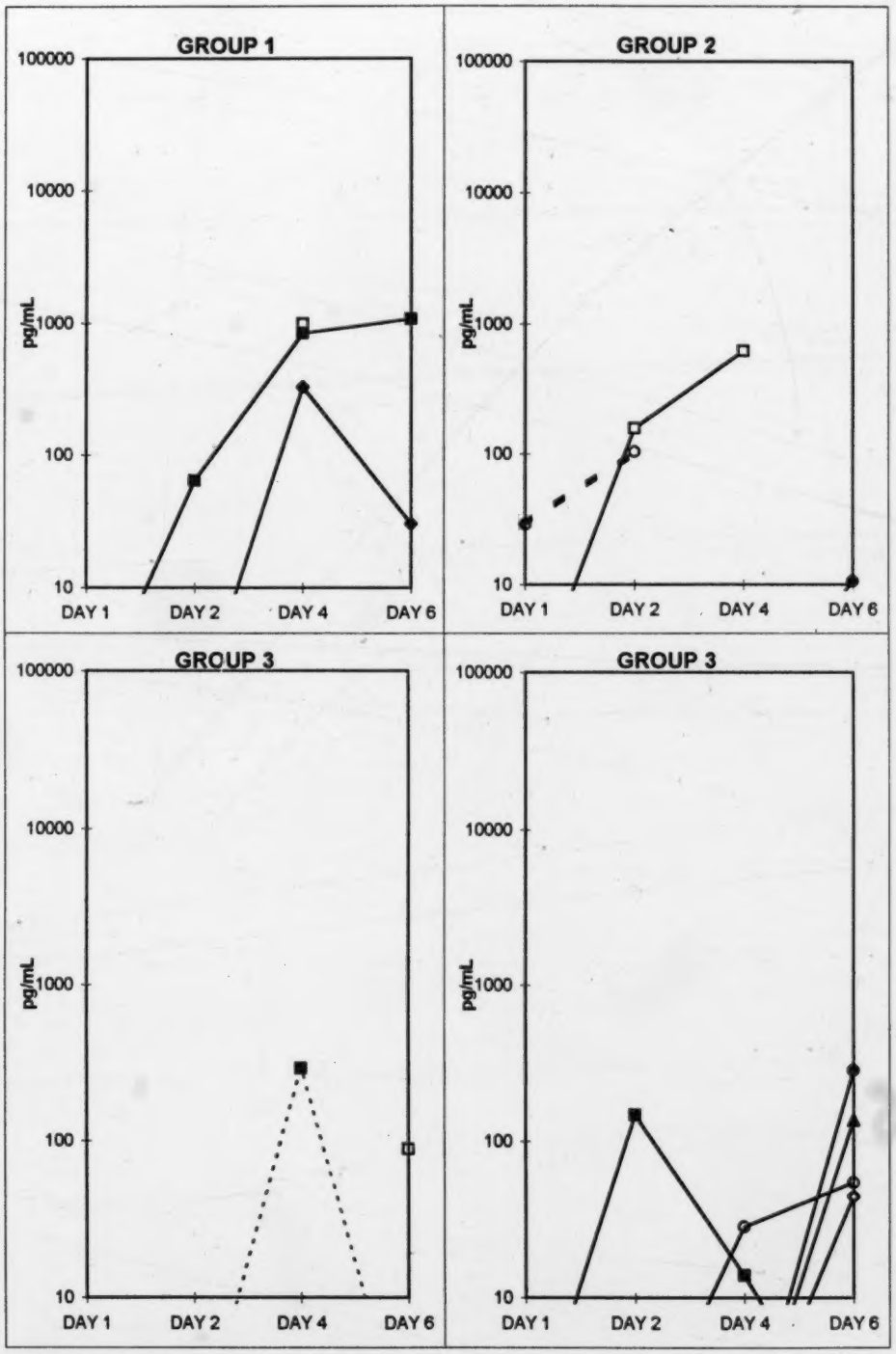


Figure 3-22. IFNγ production by PBMC upon stimulation with candida.

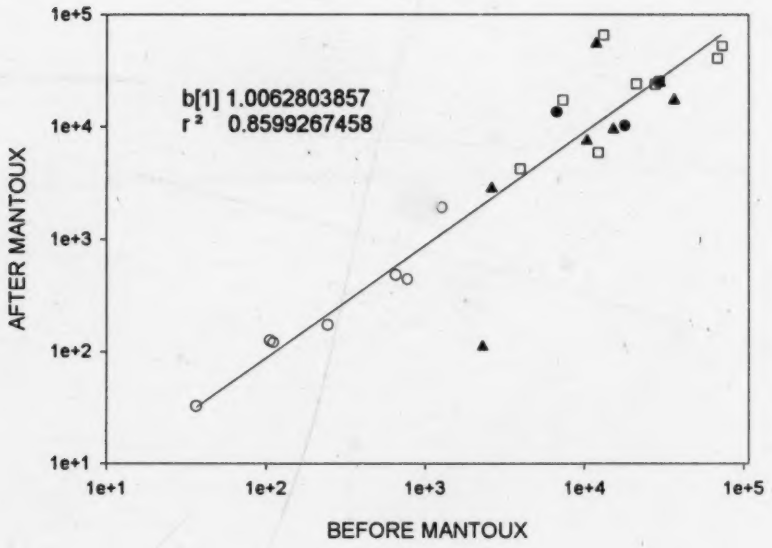


FIGURE 3-23. IFNγ production upon stimulation with 10 ug/mL of PHA. Legend:

○ Day 1 ● Day 2 □ Day 4 ▲ Day 6

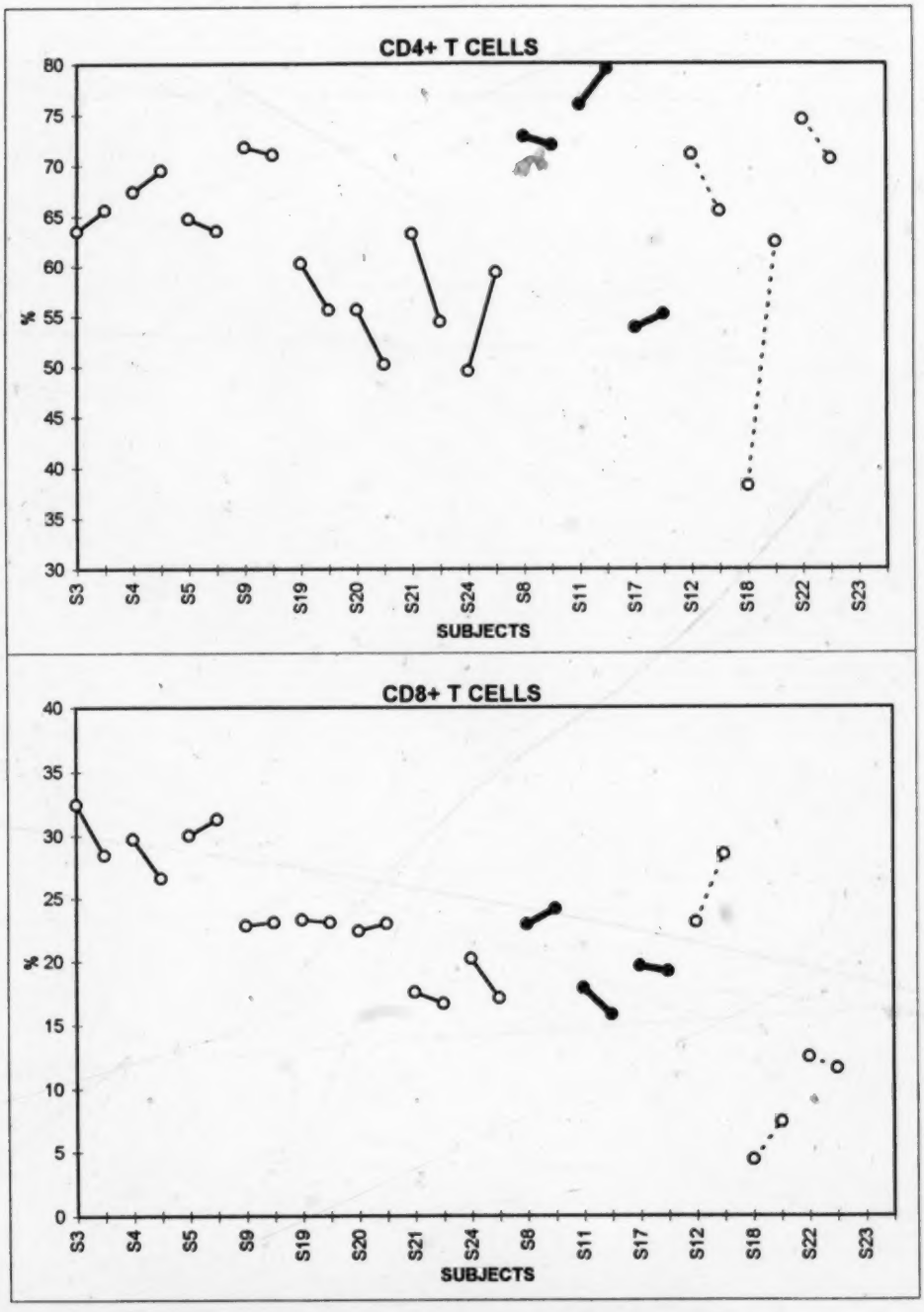


Figure 3-24. Percentage of CD4+ and CD8+ T cells in the PBMC samples before and after Mantoux.Group 1,solid circles; group 2, open circles with interrupted line; group 3, open circles with solid line.For each line, the start point means the A sample (before Mantoux test) and the end point means the B sample (after Mantoux).

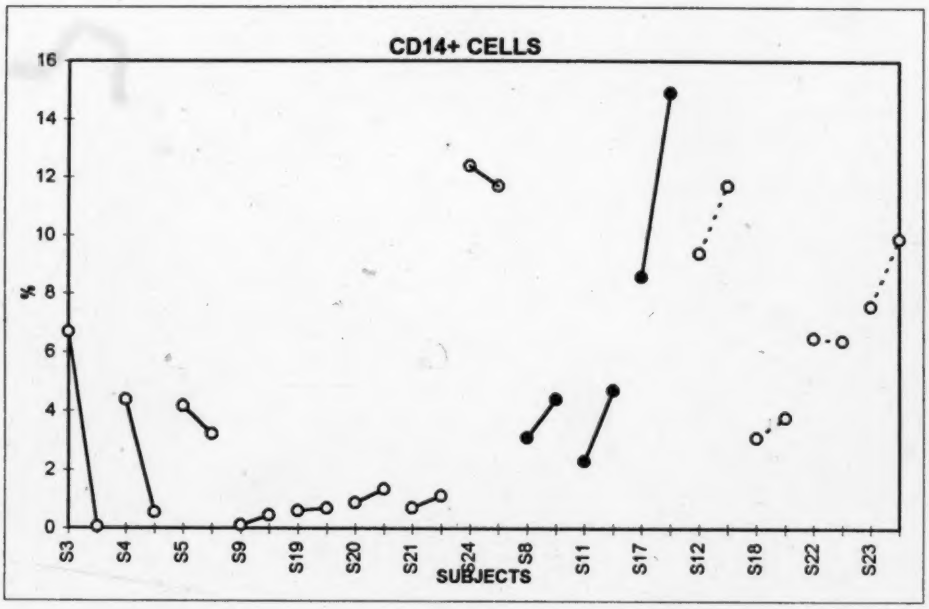


Figure 3-25. Percentage of CD14+ cells in the PBMC samples before and after Mantoux. Group 1, solid circles; group 2, open circles with interrupted line; group 3, open circles with solid line.For each line, the start point means the A sample (before Mantoux test) and the end point means the B sample (after Mantoux).

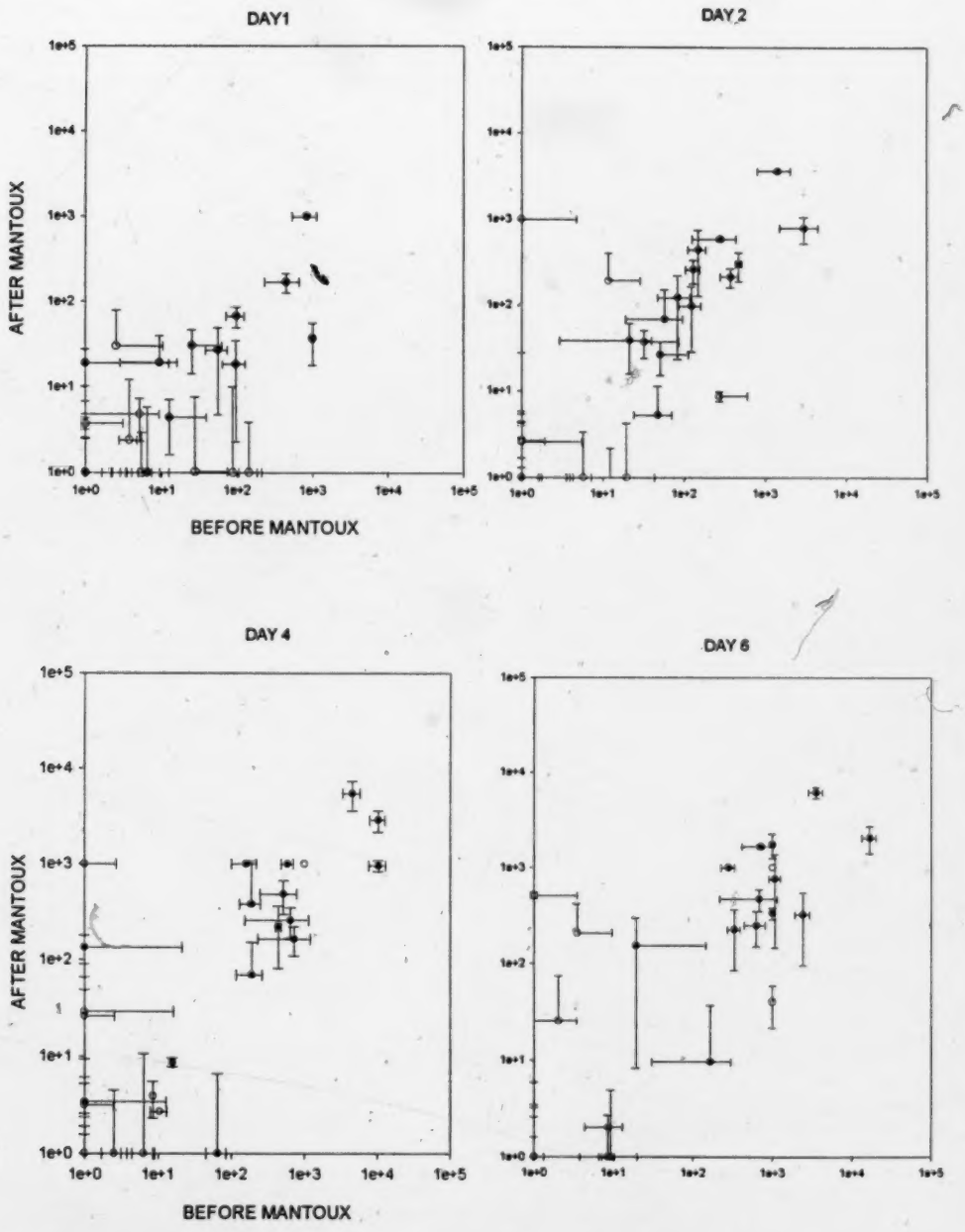


Figure 3-26 . IFNγ production by PBMC upon stimulation with 50 ug/mL of PPD (solid circles) and without antigenic stimulation (open circles) before and after Mantoux. Bars are the standard deviation of triplicates.

4. DISCUSSION AND CONCLUSIONS

4.1. DISCUSSION

The ELISA technique was chosen to measure cytokines in this work due to the previous experience in our laboratory with this method and also because it is relatively simple and quick. The large amount of samples generated by the study and the complexity of the design (different days, different antigens and different antigen concentrations) would have made the use of commercial ELISA kits very expensive. Therefore the development of an in-house ELISA was necessary. The antibodies and recombinant cytokines were commercially purchased and the assays were optimized in our laboratory. An IL2 and an IL12 assay were also tried but they proved to be more difficult to develop and were abandoned. Many conditions were evaluated as described in the Materials and Methods section. The antibody concentrations suggested by the manufacturers were substantially decreased for most assays. The manufacturer had suggested for IFNγ antibodies concentrations of 2.5 and 0.75 μg/mL for the first and second antibody respectively and they were optimized to 1 and 0.05 μg/mL. In the IL5 assay the suggested concentrations for the first and second antibody (2 and 1 μg/mL) were both decreased to 0.05 μg/mL. The IL4 assay had suggested 2.5 and 0.5 μg/mL concentrations for the first and second antibody but was optimized to 0.25 and 0.025 μg/mL respectively. In the IL13 assay, the suggested second antibody concentration of 1 μg/mL could not be further

optimized without a substantial increase of the incubation time, but the first antibody concentration was decreased by half. The TNFα assay led to a four fold reduction in the suggested concentration for the first antibody and a two fold reduction for the second antibody. Overall, these optimizations allowed a substantial reduction in the cost of the assays without a loss in their sensitivity.

The Mantoux test has been used for many years as a diagnostic tool for the diagnosis of tuberculosis (Rieder, 1995). This test was developed almost hundred years ago and it was believed to have a strong correlation with infection and acquired immunity. This is not always the case and, as shown by Orme and colleagues (Orme, 1984), the DTH response can be dissociated from protection. Also, the cutaneous anergy that can be seen in tuberculosis patients with acquired immune deficiency syndrome (Pesanti, 1994) would make an *in vitro* marker of infection desirable. IFNγ has been suggested by others as such a marker (Ellner, 1997) but the relevance of IFNγ production by antigen stimulated PBMC in humans so far has not been proven. The immune response following the intradermal injection of a heterogeneous mixture of antigens such as PPD is complex. It involves the migration of different effector and helper cells to the injection site after the initial antigen processing and presentation of the multiple components present in the PPD (Tsicopoulos, 1992). There appears to be no previous work on the immunologic consequences of the Mantoux test on cytokine production by circulating peripheral mononuclear cells.

Studies of serial tuberculin testing have suggested an increase of skin test reactivity over time (Horowitz, 1995). In that survey, individuals were

grouped by classic "Mantoux positive" and "negative" criteria. Since this is an arbitrary distinction based on clinical experience, the present study considered any induration induced by Mantoux testing as positive. The classification of "positivity" is a problem since some researchers use 10 mm as a cut off for positivity and others use a 5 mm cut off (Rieder, 1995). In the belief that any induration could mean a past encounter with mycobacterial antigens, the inclusion criteria of *any* induration for the Mantoux positive group was established. Additionally, the induration was measured in both a horizontal as well as a vertical axis since these would give a better idea of the size of the DTH reaction in contrast to the traditionally measurement of the transverse axis. Unfortunately, the difficulty in subject recruitment in the present study resulted in only two examples of Mantoux reactions (9x9 and 18x18 mm). The negative group was further split into two according to history of contact with tuberculosis, in order to explore whether cytokines measurement in the Mantoux tested subjects were more sensitive than the Mantoux test alone. The initial project was designed as a larger project and the three groups were to have further divisions, such as past BCG or past Mantoux tests. The subjects were to be followed for a period of two years with future bleedings every three months. The effect of multiple Mantoux tests over time on the expression of delayed type hypersensitivity also was to be evaluated. At the time the project was stopped, 24 subjects had a total of 55 blood samples drawn. For the purposes of this thesis all the subjects were included who had blood taken before and 48 hours after the Mantoux test. Even though the present study did not find any significant

changes in any of the immune parameters measured 48 hours after Mantoux testing, probably due to the small size of the three groups, there were many other interesting and useful findings.

The experimental design used in my experiments allows the kinetic evaluation of the amount of cytokines released into the culture media. This information not only can be used for the optimization of culture conditions but also provides some suggestions as to the cellular components involved in the response. Some individuals had a high IFNγ response even on the first day of culture. It could be speculated that this is due to readily activated memory cells, since these cells appear to exist in a continuously dividing cell population maintained in the G1 stage rather than out of cell cycle (Stout, 1992). Another explanation for such an early IFNγ production in culture could be a possible superantigen in PPD. The presence of a superantigen in M.tb was suggested by some researchers (Ohmen, 1994). They found a considerable increase of $v\beta 8^+$ T cells at the site of tuberculous pleuritis patients. Since the most impressive difference in T cell response between a superantigen and a nominal antigen is the rate of induction of the response, the early IFNγ production in PPD-stimulated cultures by some individuals could be due to the fact that these patients have the "right" vβ repertoire 'needed' by the superantigen. Moreover, PBMC stimulated with staphylococcal enteroxin B, a known superantigen, showed IFNγ levels higher than 500 pg/mL in supernatants from the first day of culture (Hayward, 1994).

The higher IFNγ production seen at days four and six of culture could represent the *in vivo* TH1 response or could be due just to a TH1 *in vitro* differentiation.

The PPD-induced IFNγ response in the present study was extremely variable between individuals. There were subjects with a low response (< 500 pg/mL) to high doses of PPD and others with a high response to low doses of PPD (> 5000 pg/mL). The kinetics of response to different PPD concentrations, however, for a given individual, remained the same.

The IFNγ production upon PPD stimulation was not significantly affected by the Mantoux in the group of subjects tested. Even though there were subjects with lower and higher IFNγ responses to PPD, there was no significant effect by the Mantoux on these different subjects.

The Mantoux status of an individual person did not appear to affect the IFNγ response to PPD. This failure to detect a difference could be due to the small number of subjects.

IFNγ has a very important role for anti-mycobacterial activity in mice (Cooper, 1994). In humans its role is still uncertain (Barnes, 1993). Surcel and colleagues (Surcel, 1994) showed that IFNγ mRNA expression in PBMC is similar in Mantoux positive individuals and tuberculosis patients. Lin and colleagues (Lin, 1996) found a higher concentration of IFNγ in PBMC supernatants from Mantoux positive individuals when compared to tuberculosis patients. The difference of results between these two studies could be explained by the difference in methods but also could be ascribed to an inconsistency of

the IFNγ response. Even though the present study does not include TB patients, our results suggest that IFNγ production is very consistent over time and therefore a useful tool.

In addition, the optimal dose of antigen tested is important since the correlation between PPD-induced IFNγ production before and after Mantoux testing was stronger with the higher dose of PPD. However, at day six of culture this correlation is lost, probably due to culture changes. This observation brings to light the importance of the choice of "optimal" experimental conditions. Most laboratories choose the optimal day of culture based on the peak concentration of cytokine production. The present IFNγ correlations results showed that this is not always true. Although the highest production of the cytokine was observed at day 6 of culture, day 4 was the best day to show the consistency of IFNγ response. At day 6 of culture, this correlation had dropped significantly.

There were four subjects (S11, S20, S23, S24) who had high spontaneous release of IFNγ on some days of culture. No technical explanation was found. These four subjects had offscale values for IFNγ detection on some days of their control cultures. Since those days were not added to the correlation analysis for any immune parameter, it is unknown if this would impact on the results presented. Also, some individuals had increased spontaneous IFNγ release after Mantoux. These individuals were not necessarily the same who had a higher spontaneous IFNγ release previous to the Mantoux testing.

A relevant technical point in the present study was that the cytokine levels were not adjusted according to the cell number of each culture at the time

of supernatant harvesting. Also important are the limitations of the method used to detect cytokine production. The ELISA does not measure functionality of the cytokine or how many cells in the culture are responsible for its production. Bioassays of specific cytokines could assess the activity of the cytokine. However, these assays have the inconvenience of working with cell lines, such as time for propagation, risk of contamination, and they do not allow the quantification of cytokine producing cells. On the other hand, the elispot technique permits enumeration of the cells producing cytokines (Kuby, 1994). The present study used only PBMC, but it would be very interesting to identify the different cell populations responsible for cytokine production. In addition, limiting dilution analysis would be helpful to quantify the antigen specific cells.

In general, there was no difference in the TI response to PPD before and after Mantoux testing. In a study where blood was drawn at different time intervals after Mantoux testing, a depression of the TI response to PHA and PPD at 10 μg/mL in Mantoux positive individuals was found on the first day and would persist up to the seventh day (Thestrup-Pedersen, 1974). There are some technical differences between that study and the present one. The former cultured the cells in tissue culture tubes while the present study used microtitre wells. But, as shown by Young and colleagues (Young, 1995), this should not make an important difference. Also, the concentration of cells used for the PHA assay was much higher than in the present study and the harvesting was done at day six. Previous work done in our laboratory showed that the PHA response at these conditions is far from optimal and there is an unpredictable variation to

the PHA-induced TI response due probably to cell death. Other workers also showed that PHA-induced TI falls markedly after day four and this is probably due to the drop in accessory cells in the culture (Ijichi, 1996). Although some persons showed some decrease or increase to the PPD-induced TI response after Mantoux in the present study, this was not a dramatic change. However, these different results in the two studies could be due to the small number of Mantoux positive subjects in the present study compared with 10 Mantoux positive individuals in the study by Thestrup-Pedersen et al.. Also, these investigators used 1 TU of PPD for the Mantoux test whilst we used the standard 5 TU. In addition, the present study found a correlation between the PPD- induced TI response before and after Mantoux testing. This correlation was better at the intermediate dose (10 μg/mL) of PPD but was also significant at the higher PPD dose (50 μg/mL).

It was also found that some individuals from group three had a higher TI response to PPD in comparison with other individuals from the same group. This finding could mean a higher sensitivity of the TI response to past exposure to mycobacteria or just a biological variation. Since these persons did not present any different pattern in relation to the other immune parameters measured, the clarification of this finding would need the testing of a larger number of subjects.

A very interesting finding was the correlation between the TI and the IFNγ response to PPD. The PPD-induced IFNγ production before and after Mantoux had a better correlation at day four than other days, the correlation with the intermediate dose PPD-induced TI response was higher at day six. The following

may explain these findings. The correlation was higher between the TI and the IFNγ response with the intermediate PPD dose probably due to the fact that this concentration was the best to identify a consistency in the TI response. Also, in the calculations for the correlations of PPD-induced IFNγ production before and after Mantoux, points were not included if either the before or after Mantoux value was offscale. However, for the calculations for the correlations of PPD-induced IFNγ production and TI response, all on-scale PPD-induced IFNγ production were included. Since most of the offscale values were within the higher PPD concentration, these data should not affect the higher correlation found at day 6 between PPD-induced IFNγ production and TI response.

Another aspect is that IFNγ is generally accepted as a TH1 differentiation factor and not as a major proliferative cytokine (Kuby, 1994). It could be speculated that, since both responses are so well correlated, they are both affected by a same third factor. This third factor could be IL2 since it is known that TI is well correlated with soluble IL2 receptor production, and experiments with addition of neutralizing anti-IL2 receptor to PPD-stimulated PBMC show a significant inhibition of IFNγ production (Ota, 1990).

The recall response by tetanus toxoid and Candida were overall low and apparently not affected by the Mantoux test in any of the immune parameters evaluated. Since most subjects had a low response to tetanus toxoid because their vaccination had been many years earlier, it would have been interesting to have these subjects revaccinated before starting the study.

PPD-induced IL4 or IL5 were produced by only a few subjects. These results are in agreement with the assumed TH1 nature of PPD stimulation, since it originates from an intracellular bacterium. In this study, most subjects also did not produce IL13 in response to PPD stimulation which is also in agreement with the thought by some researchers of IL13 as a TH2 cytokine (Lebel-Binay, 1995) and with the evidence that IL4 and IL13 have many properties in common (Nilsson, 1995). TNFα was produced with all concentrations of PPD tested in a dose dependent manner. The early peak at day one is in agreement with a macrophage origin for this cytokine. The production of TNFα, a cytokine involved in the TH1 response seen at the site of human tuberculosis (Barnes,1990), is known to be important in the stimulated production of IFNγ by natural killer cells (Andersen, 1997). Some individuals showed another peak of PPD-stimulated TNFα response at day six.

The PHA response was used to indicate the overall viability of the samples and any general immune suppression. All subjects had a good PHA-induced IFNγ response. Many subjects had offscale values already at day one of culture and the values remained or rose further (many offscale). This was probably due to the polyclonal activation characteristic of this mitogen. The overall PHA-induced IFNγ response was not affected by the Mantoux test. All TH2 cytokines tested were present in supernatants from PHA-stimulated cells. This study showed that a polyclonal response generates high levels of both TH1 and TH2 cytokines.

Finally, immunophenotyping of CD4 and $CD8^+$ T cells did not show any significant differences between the groups, neither before nor after Mantoux testing. The limitations of the method, such as demarcation of the lymphocyte gate, make it necessary to use a larger number of samples to be able to detect any difference. Nonetheless, cells positive for CD14, a known macrophage marker, showed a trend towards an increase in groups one and two. In an *in vitro* model it was shown that monocytes undergo spontaneous apoptosis in unstimulated cultures and, at the same time, this is avoided in M.tb. infected monocyte cultures. The addition of exogenous TNFα to the unstimulated monocyte cultures can prevent apoptosis and this effect can be inhibited by supplementing the cultures with neutralizing anti-TNFα (Durbaum-Landmann, 1996). It is tempting to associate the higher number of monocytes in Mantoux positive (group 1) and TB contacts (group 2) with the higher capacity for TNFα production in these groups. Unfortunately, the study does not have enough data for such a conclusion.

4.2. CONCLUSIONS

1. There is IFNγ production upon PPD stimulation in all subjects tested.
2. There is a large variation of IFNγ production between the different subjects.
3. There is no significant difference before and after Mantoux testing in IFNγ production upon PPD stimulation.

4. The amounts of IFNγ produced by Mantoux-positive subjects were within the range of the values for Mantoux-negative subjects.

5. The IFNγ response upon PPD stimulation is consistent for the same individual.

6. The TI response to PPD stimulation is not significantly affected after 48 hours of Mantoux testing in the subjects tested and is consistent for a given subject.

7. There is a strong correlation between the IFNγ and TI responses upon stimulation with PPD.

Overall, these results suggest that the Mantoux reaction does not change the IFNγ or the TI response to antigenic stimulation of circulating peripheral blood mononuclear cells. IFNγ and TI are strongly correlated and consistent over time. A larger subject sample is needed to identify any distinct pattern between groups with different Mantoux status.

5. REFERENCES

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6. Appendix

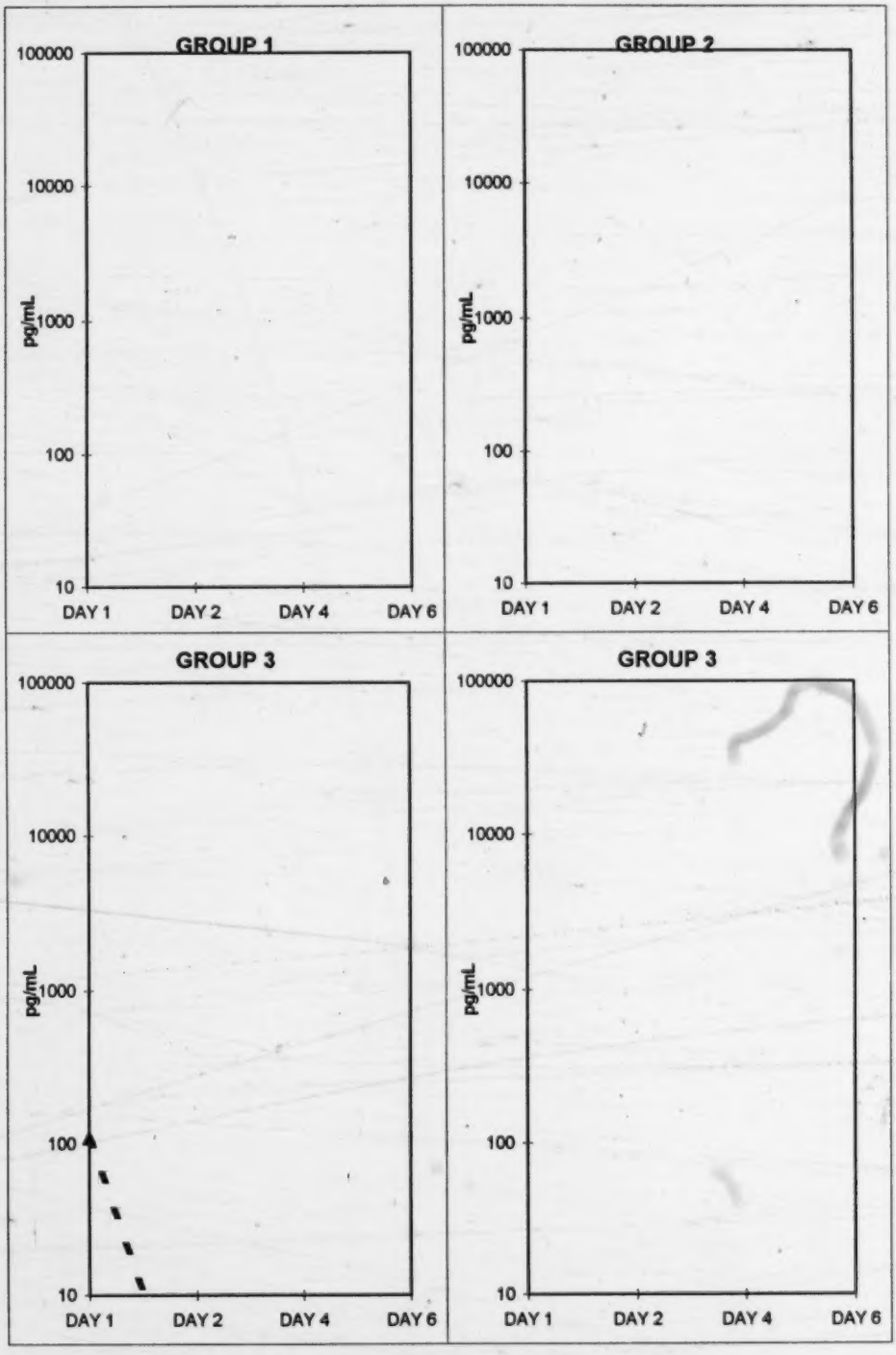
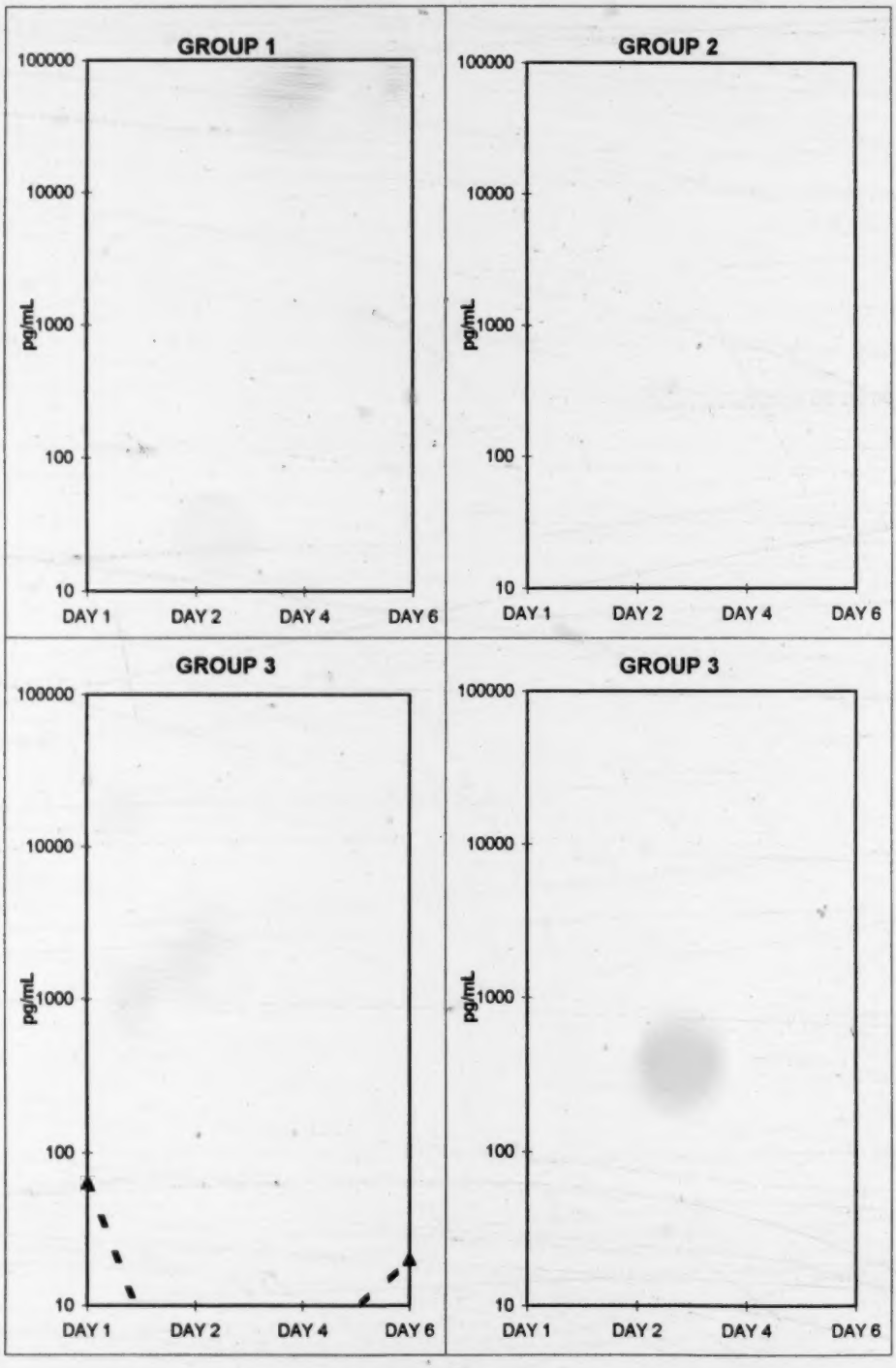


Figure A-1. IL4 production by PBMC upon stimulation with 10 ug/mL of PPD.



FigureA-2. IL4 production by PBMC upon stimulation with 50 ug/mL of PPD.

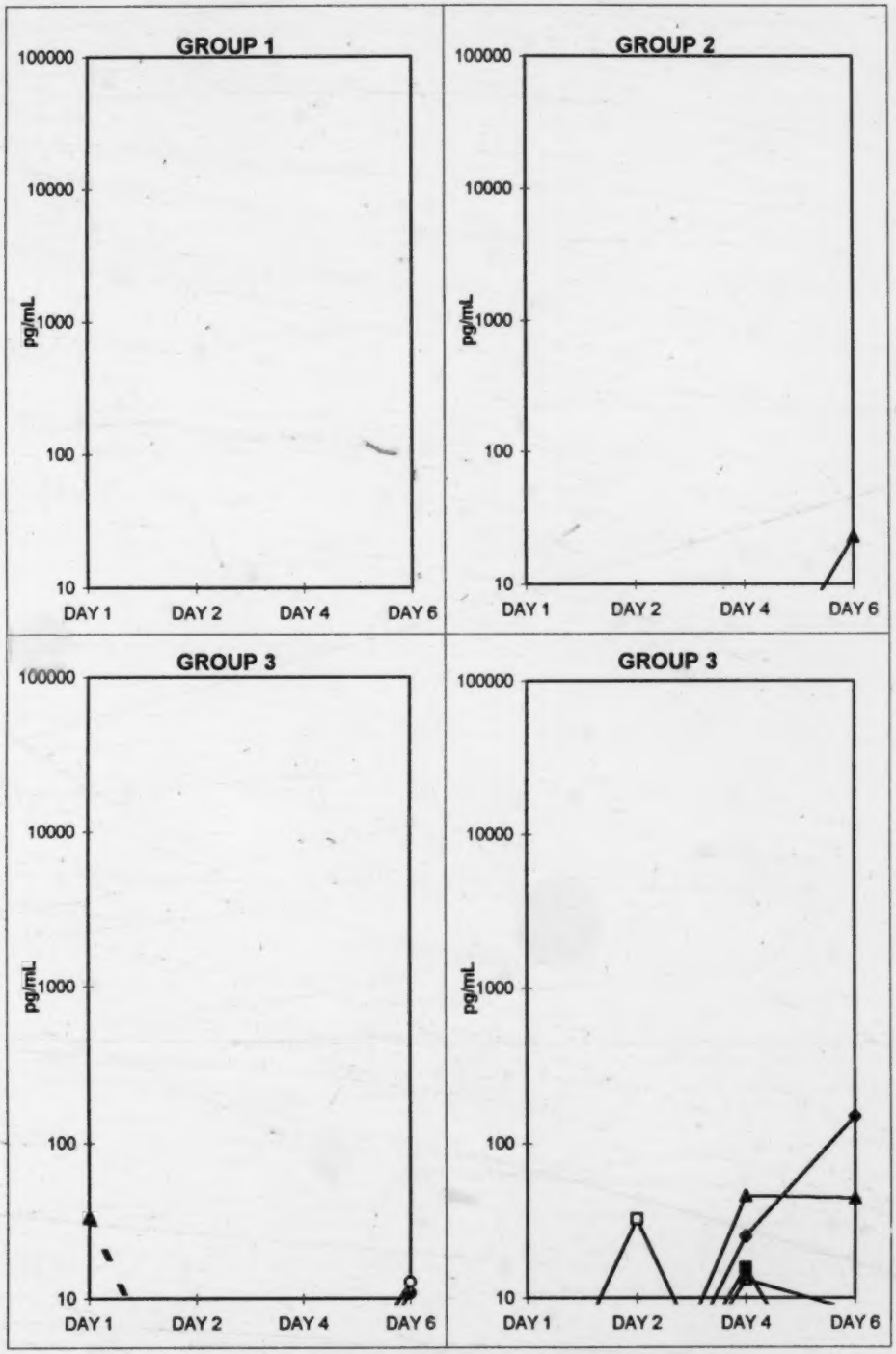
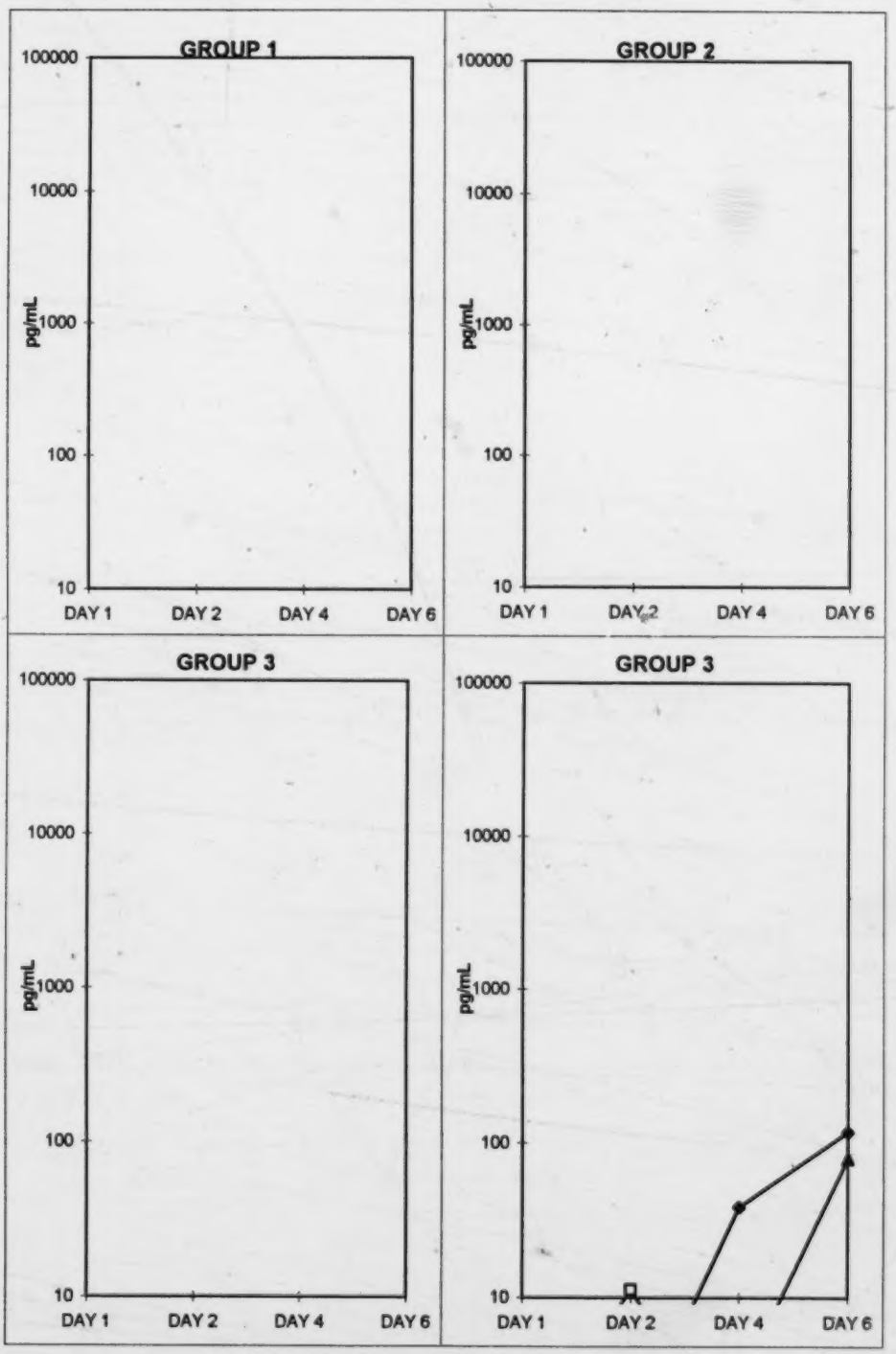


Figure A-3. IL5 production by PBMC upon stimulation with 10 ug/mL of PPD.



FigureA-4. IL5 production by PBMC upon stimulation with 2 ug/mL of PPD.

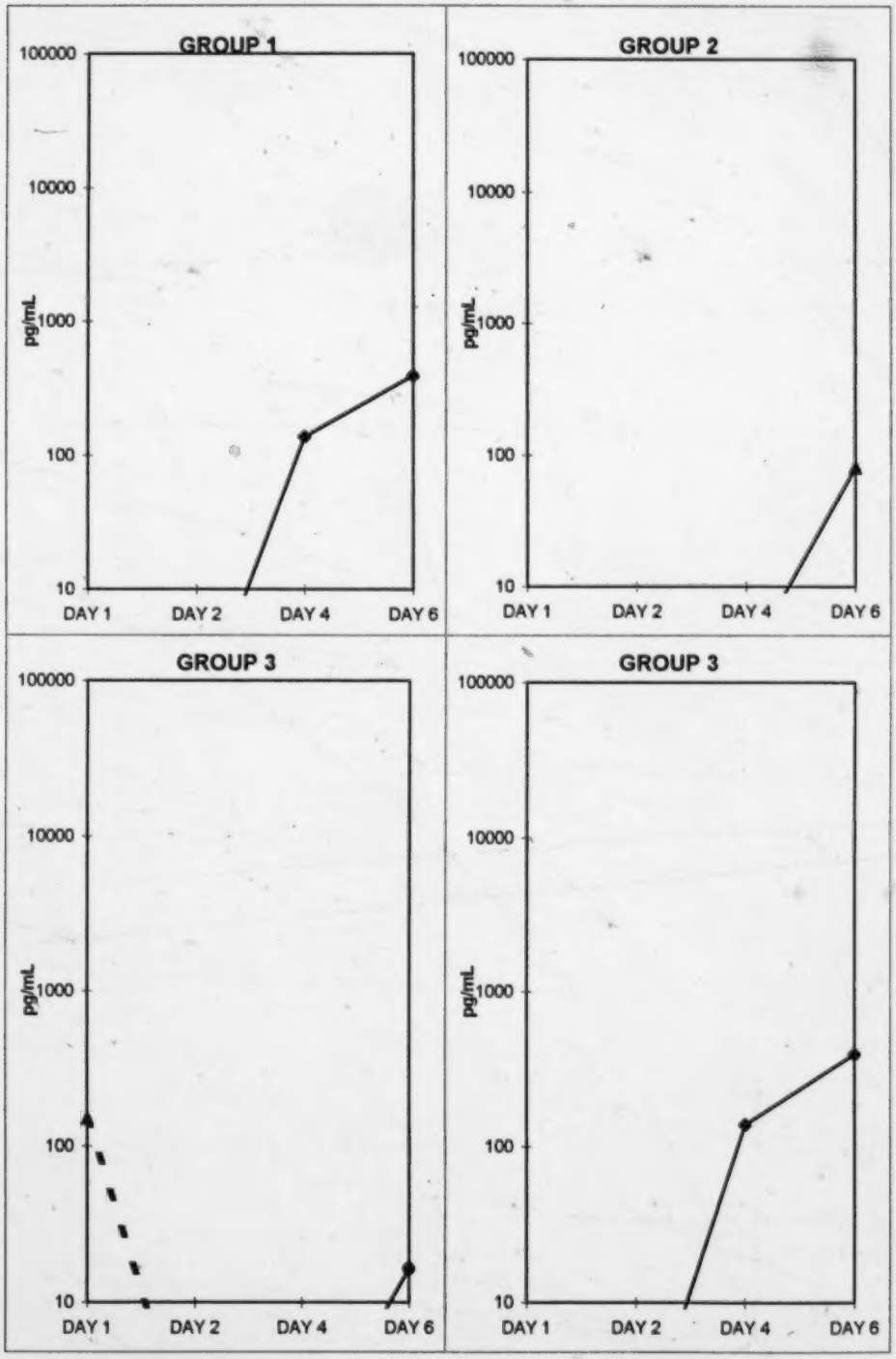


Figure A-5. IL13 production by PBMC upon stimulation with10 ug/mL of PPD.

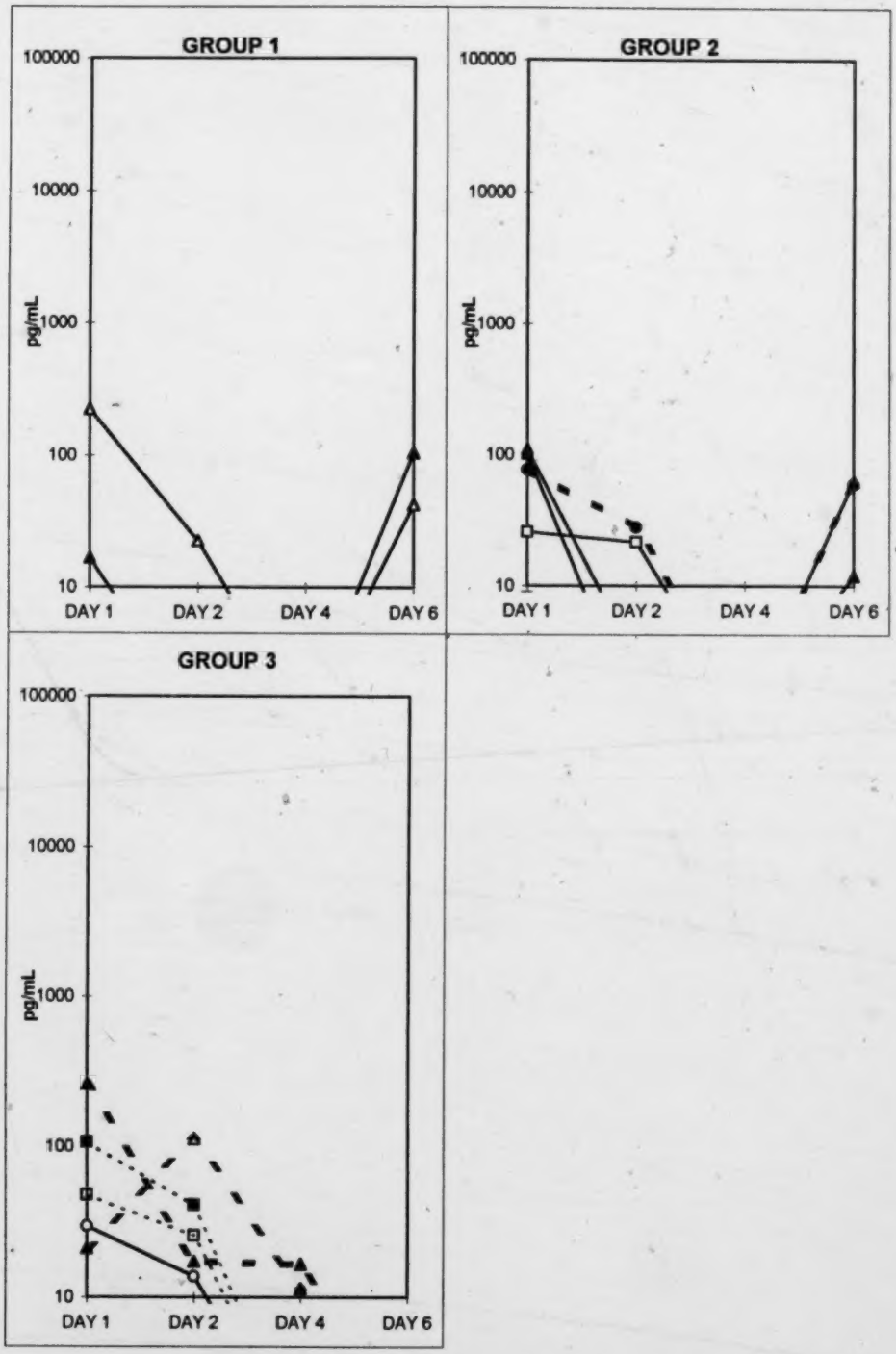


Figure A-6. TNFa production by PBMC upon stimulation with 2 ug/mL of PPD.

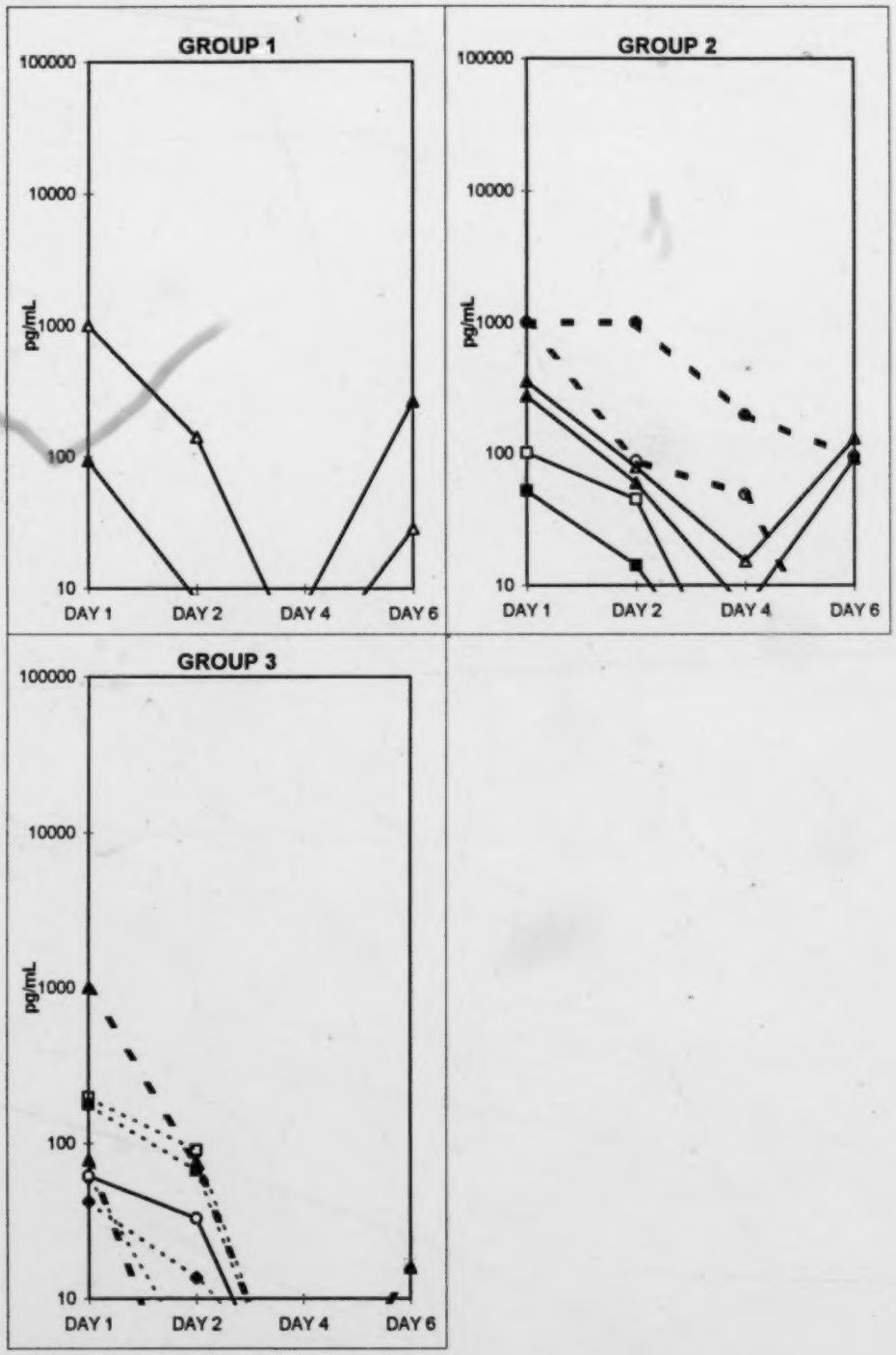


Figure A-7. TNFa production by PBMC upon stimulation with 10 ug/mL of PPD.

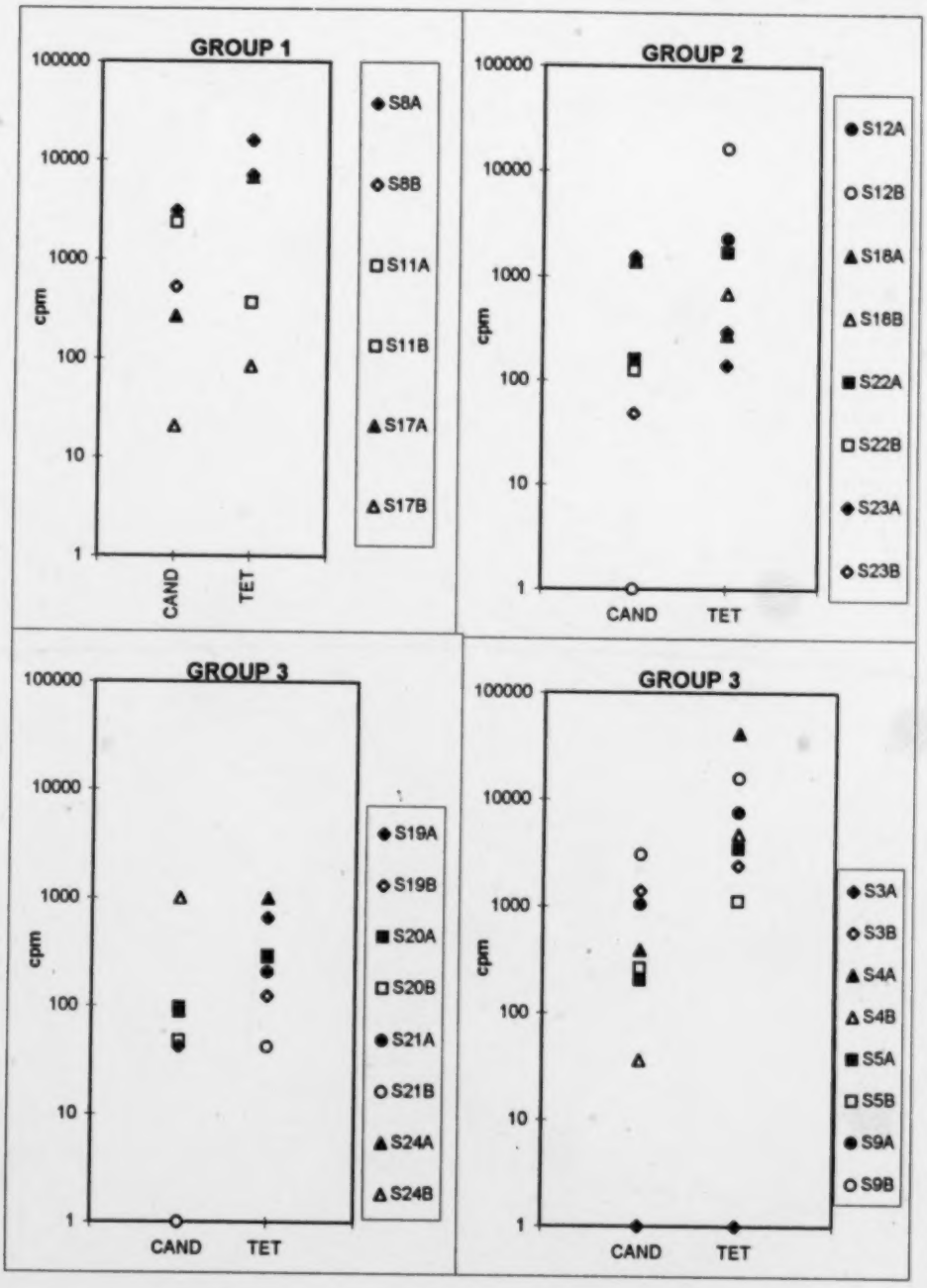
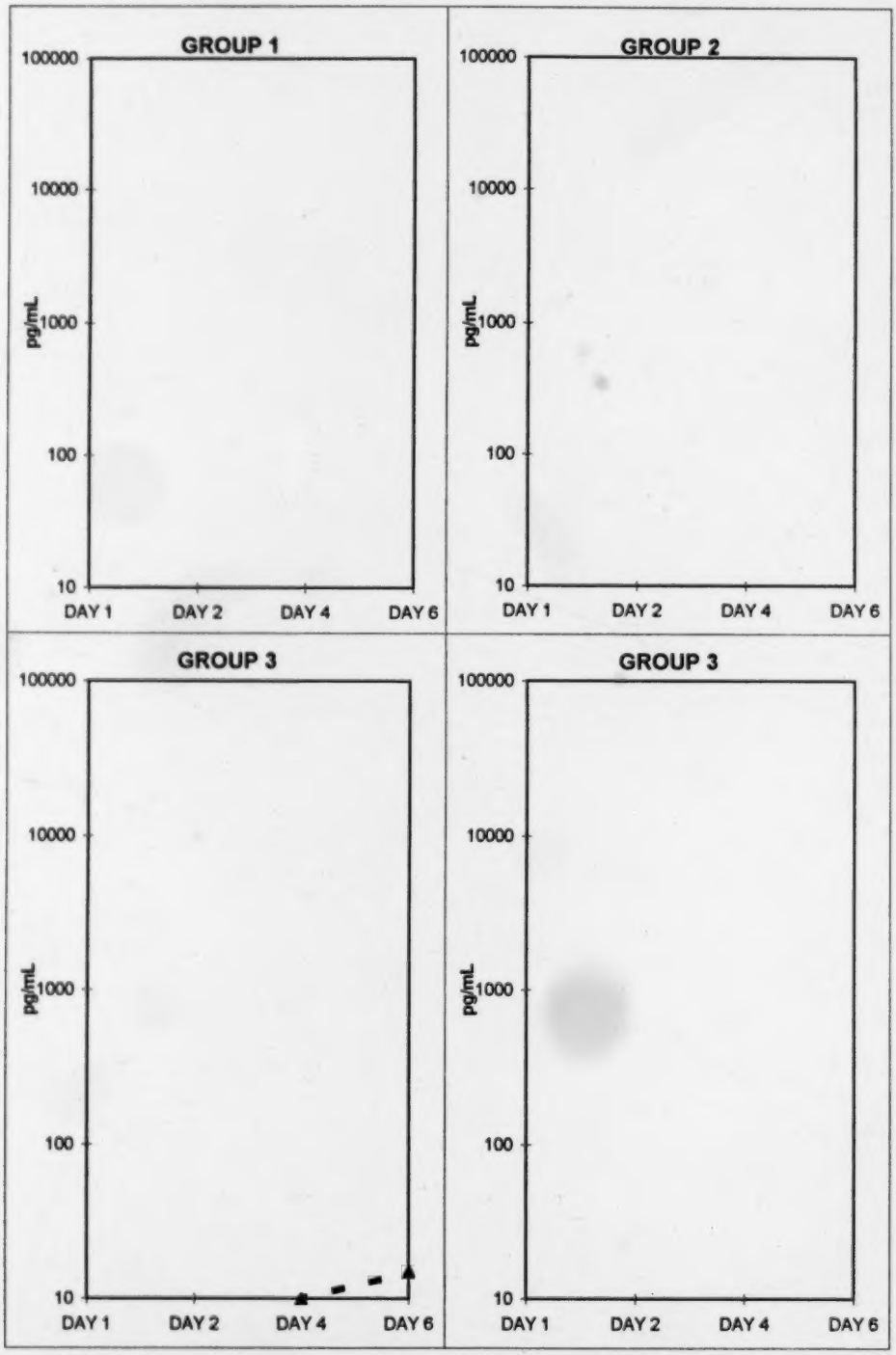


Figure A-8. TI by PBMC upon stimulation with candida and tetanus.
Results are in counts per minute (cpm).



FigureA-9. IL4 production by PBMC upon stimulation with candida.

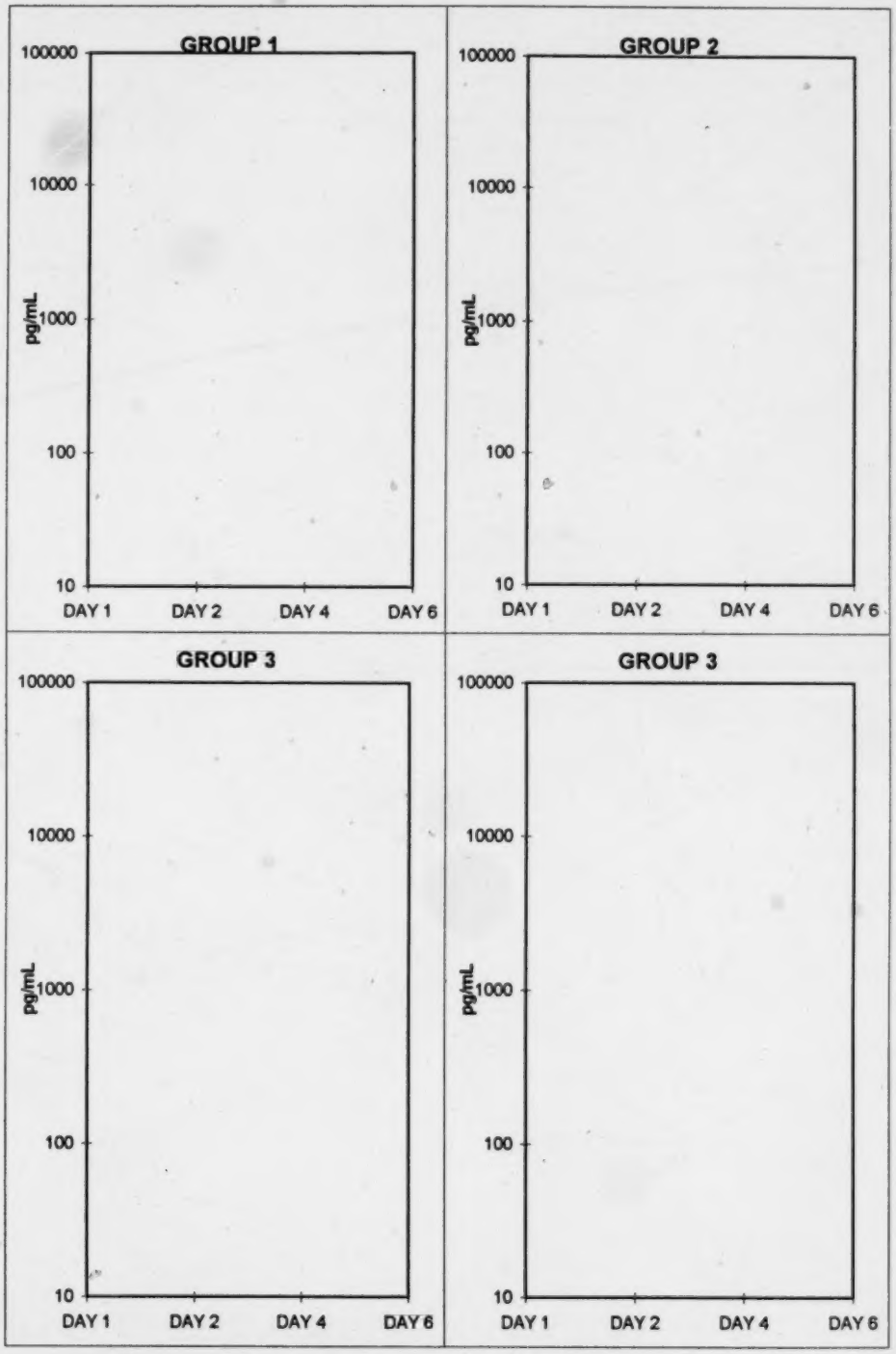


Figure A-10. IL4 production by PBMC upon stimulation with tetanus.

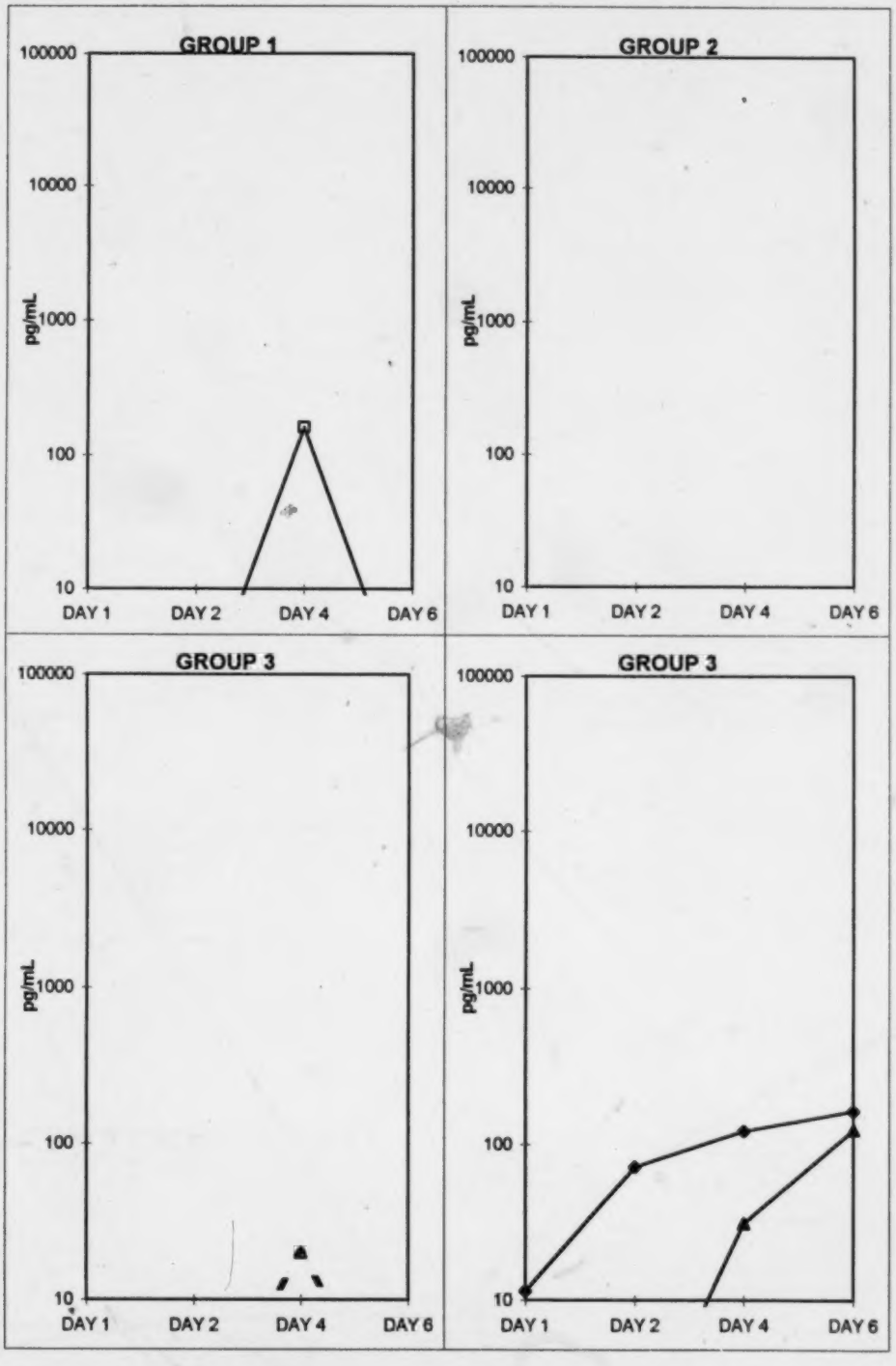


Figure A-11. IL5 production by PBMC upon stimulation with candida.

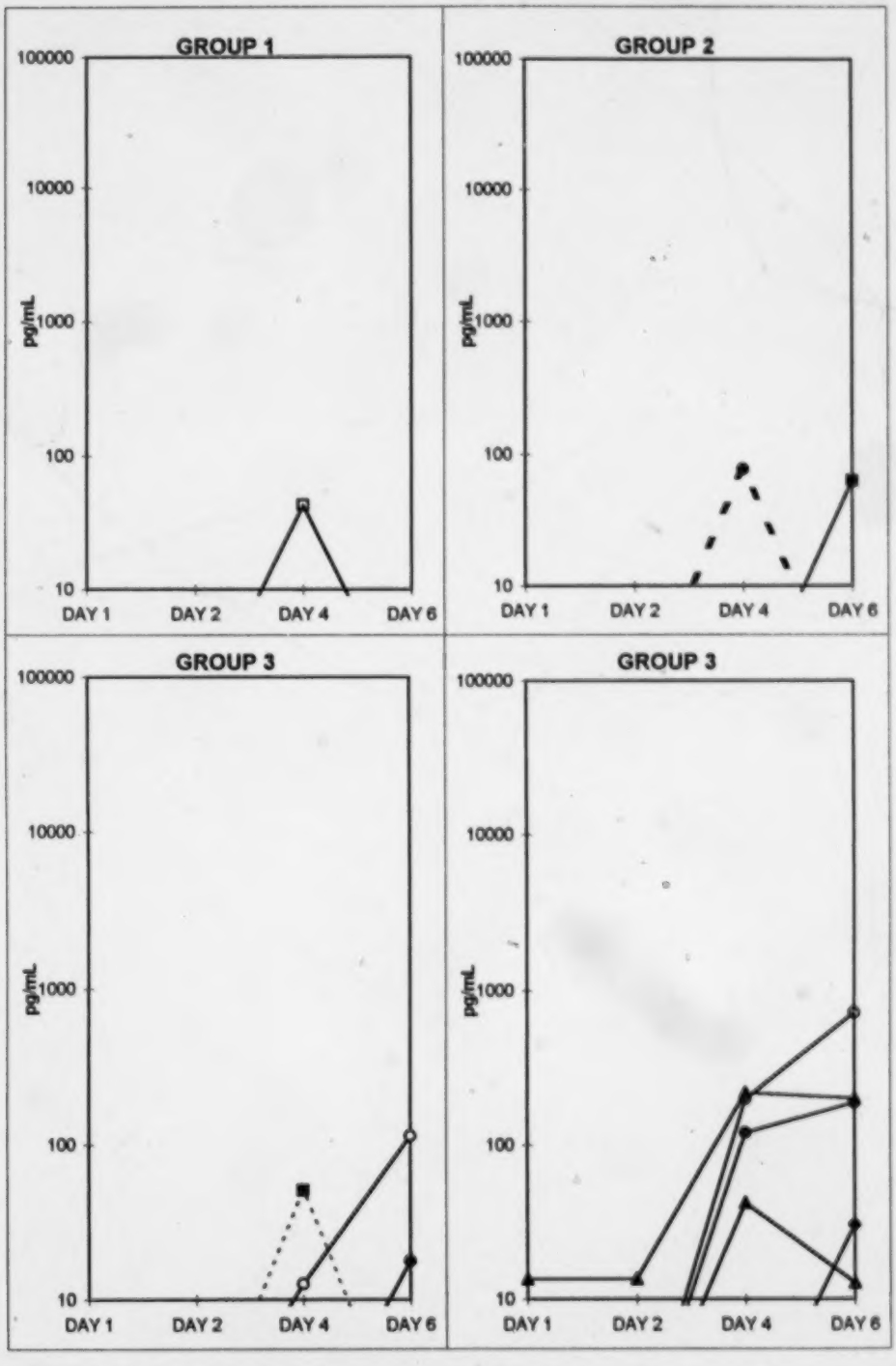
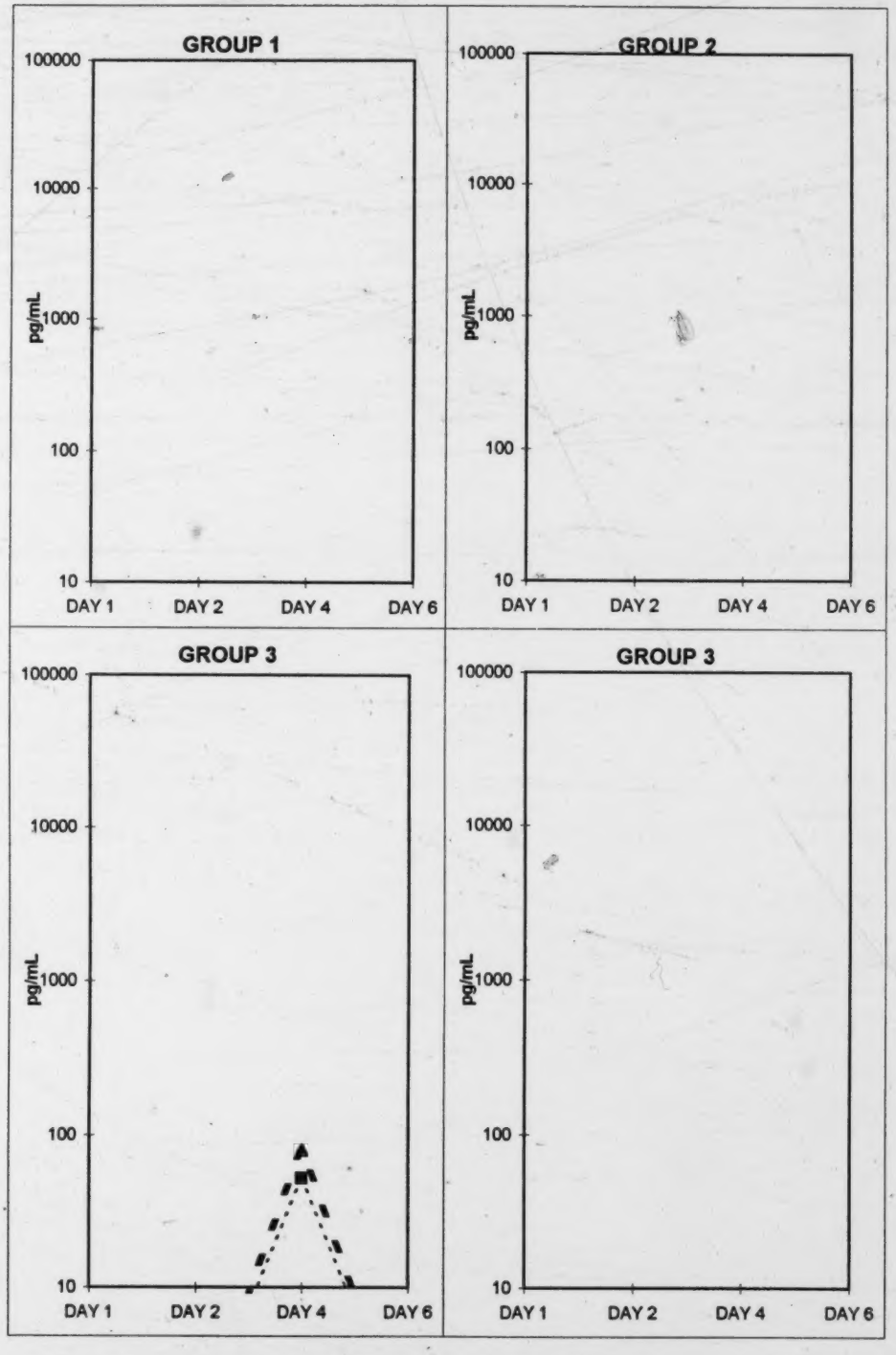


Figure A-12. IL5 production by PBMC upon stimulation with tetanus.



FigureA-13. IL13 production by PBMC upon stimulation with candida.

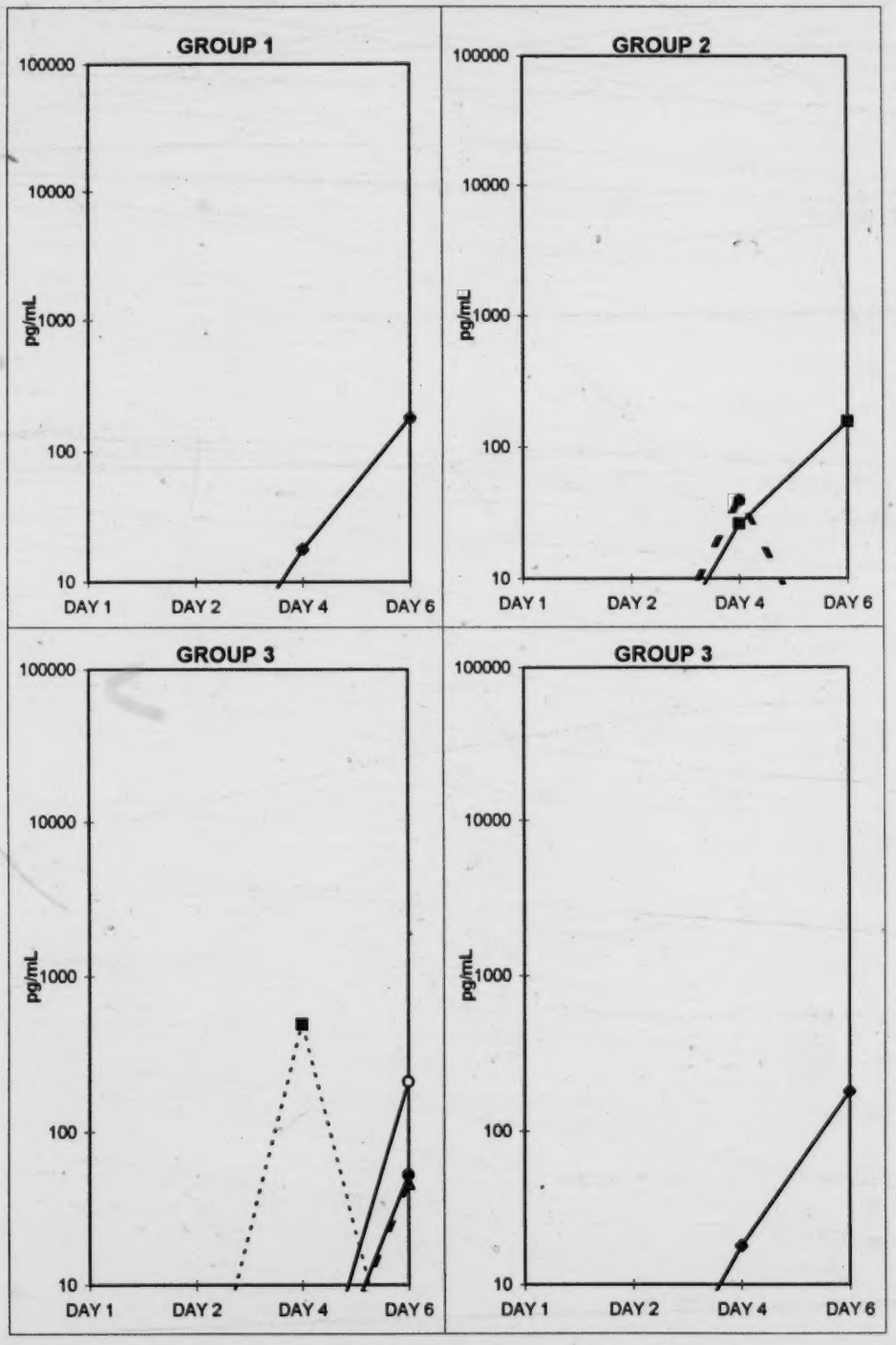
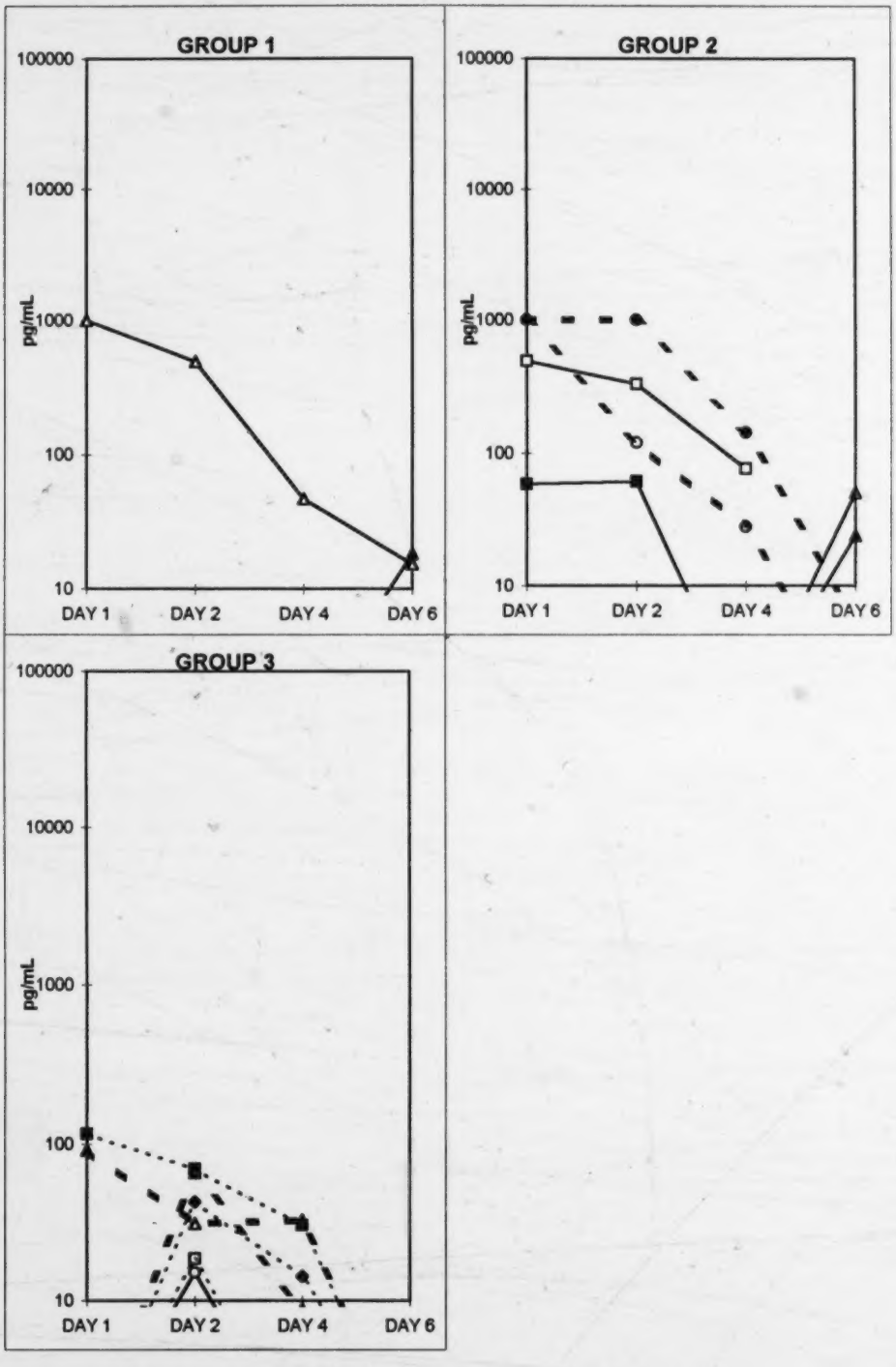


Figure A-14. IL13 production by PBMC upon stimulation with tetanus.



FigureA-15. TNFa production by PBMC upon stimulation with candida.

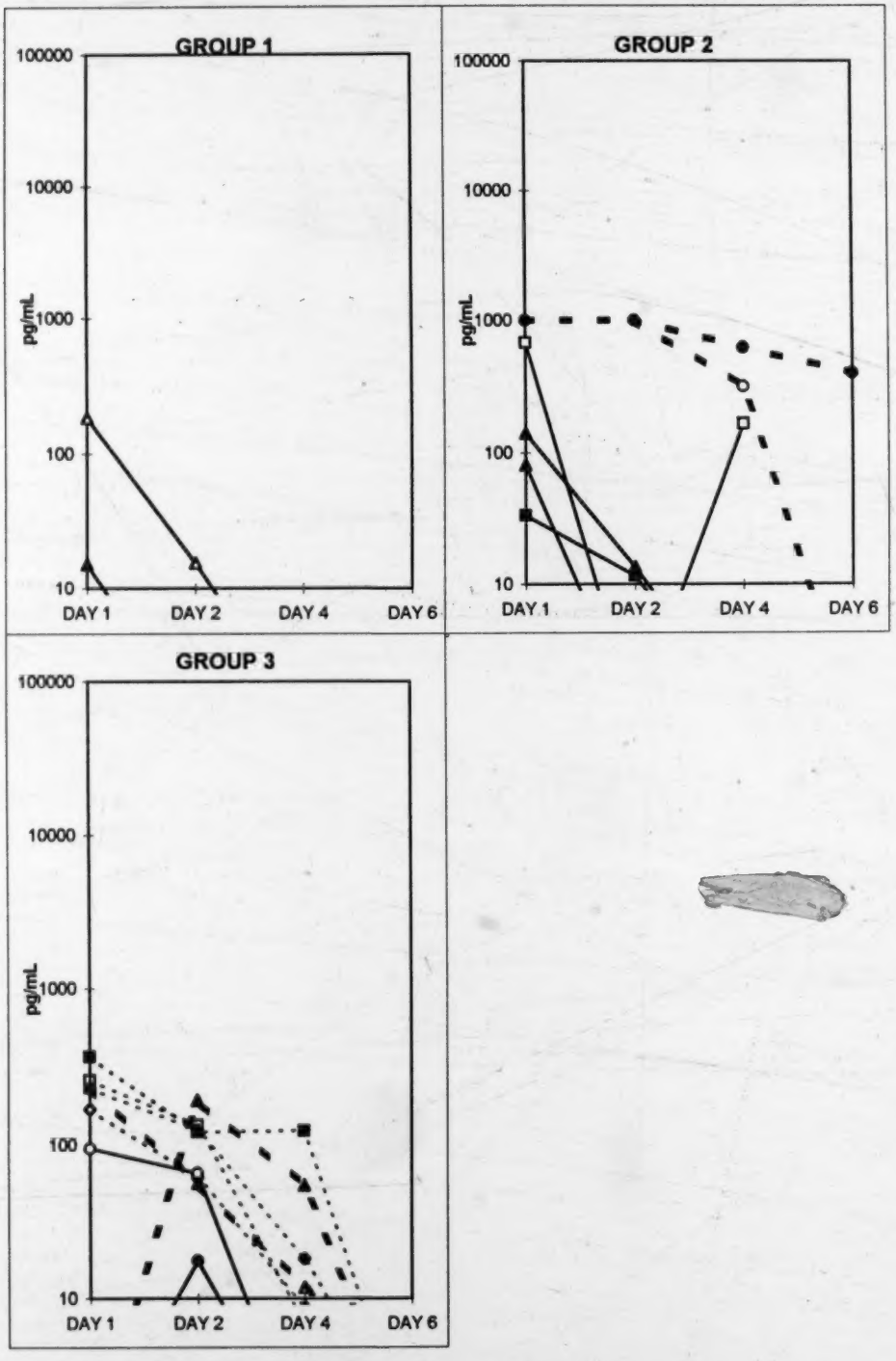


Figure A-16. TNFa production by PBMC upon stimulation with tetanus.

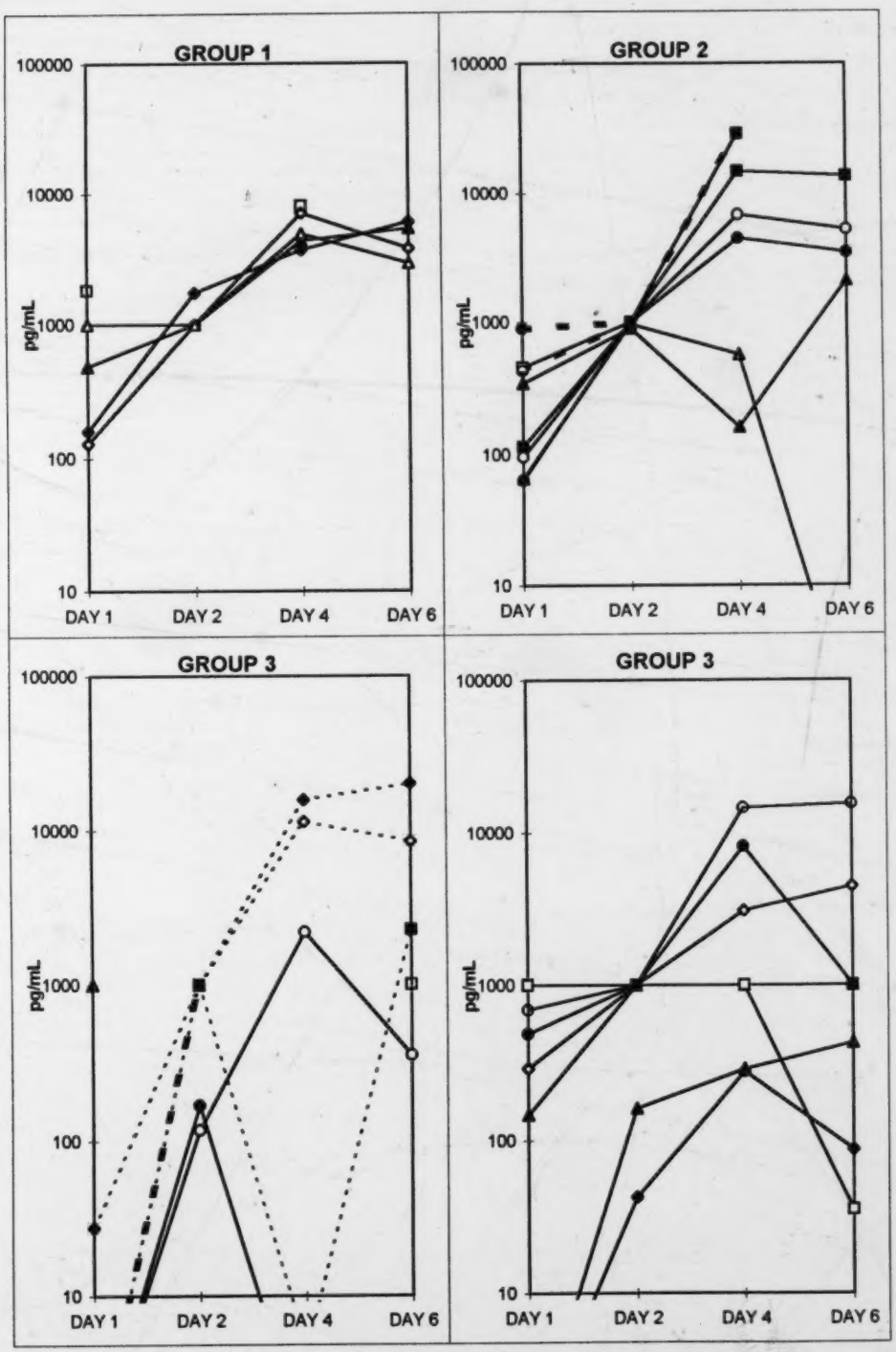


Figure A-17. IFNg production by PBMC upon stimulation with 2 ug/mL of PHA.

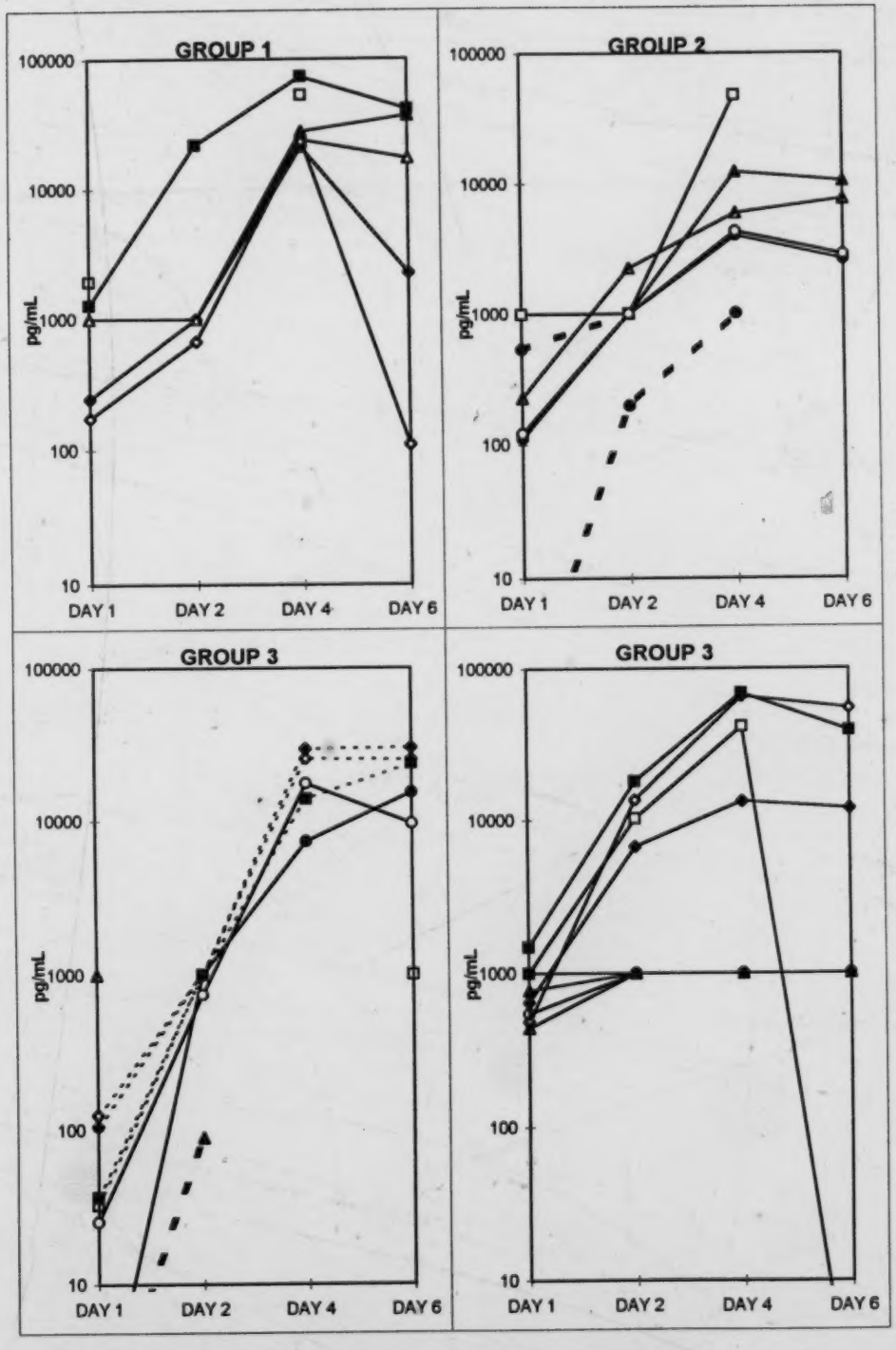
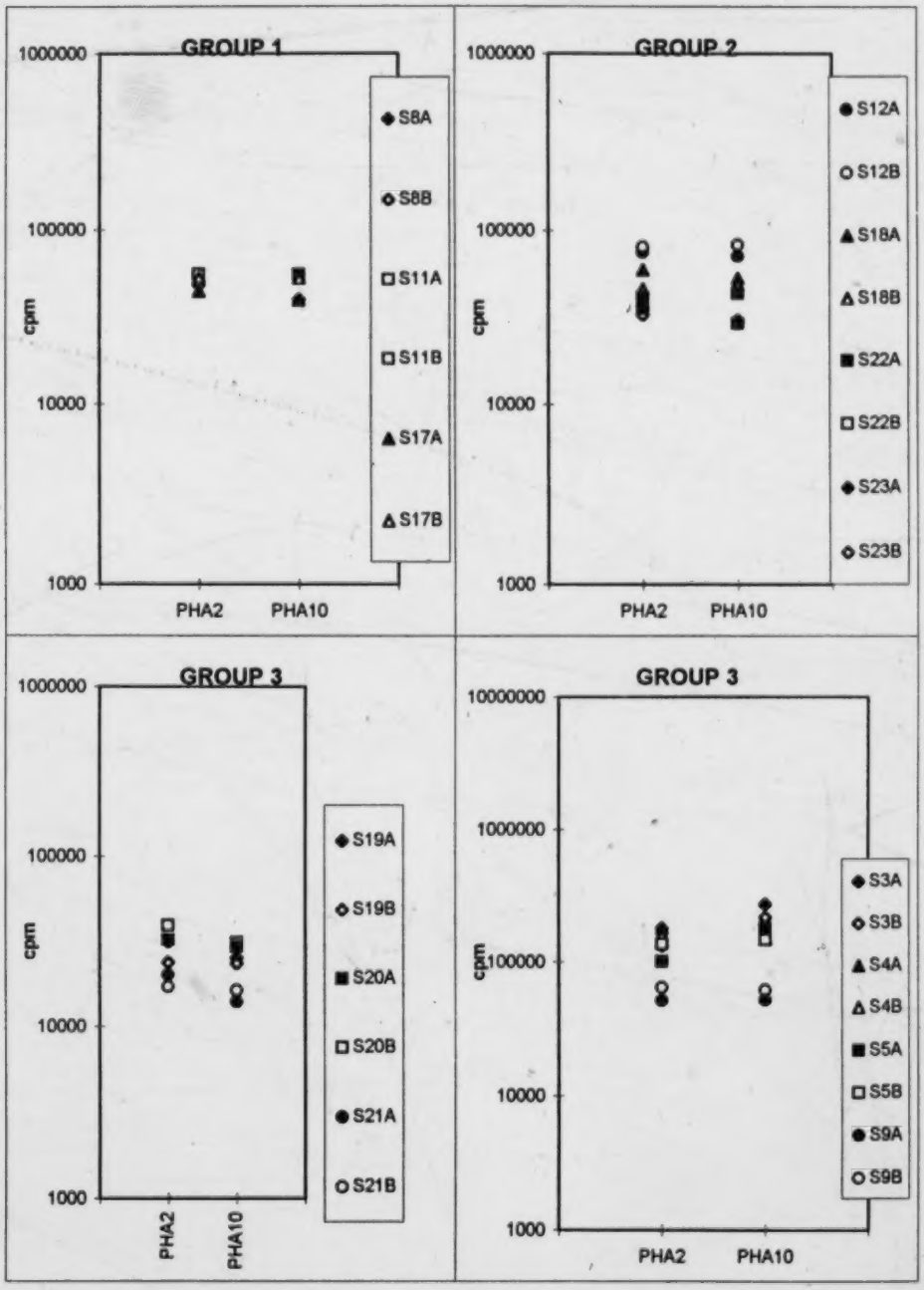


Figure A-18. IFNg production by PBMC upon stimulation with candida.



FigureA-19. TI by PBMC upon stimulation with two different doses of PHA (2 and 10 ug/mL).Results are in counts per minute (cpm).

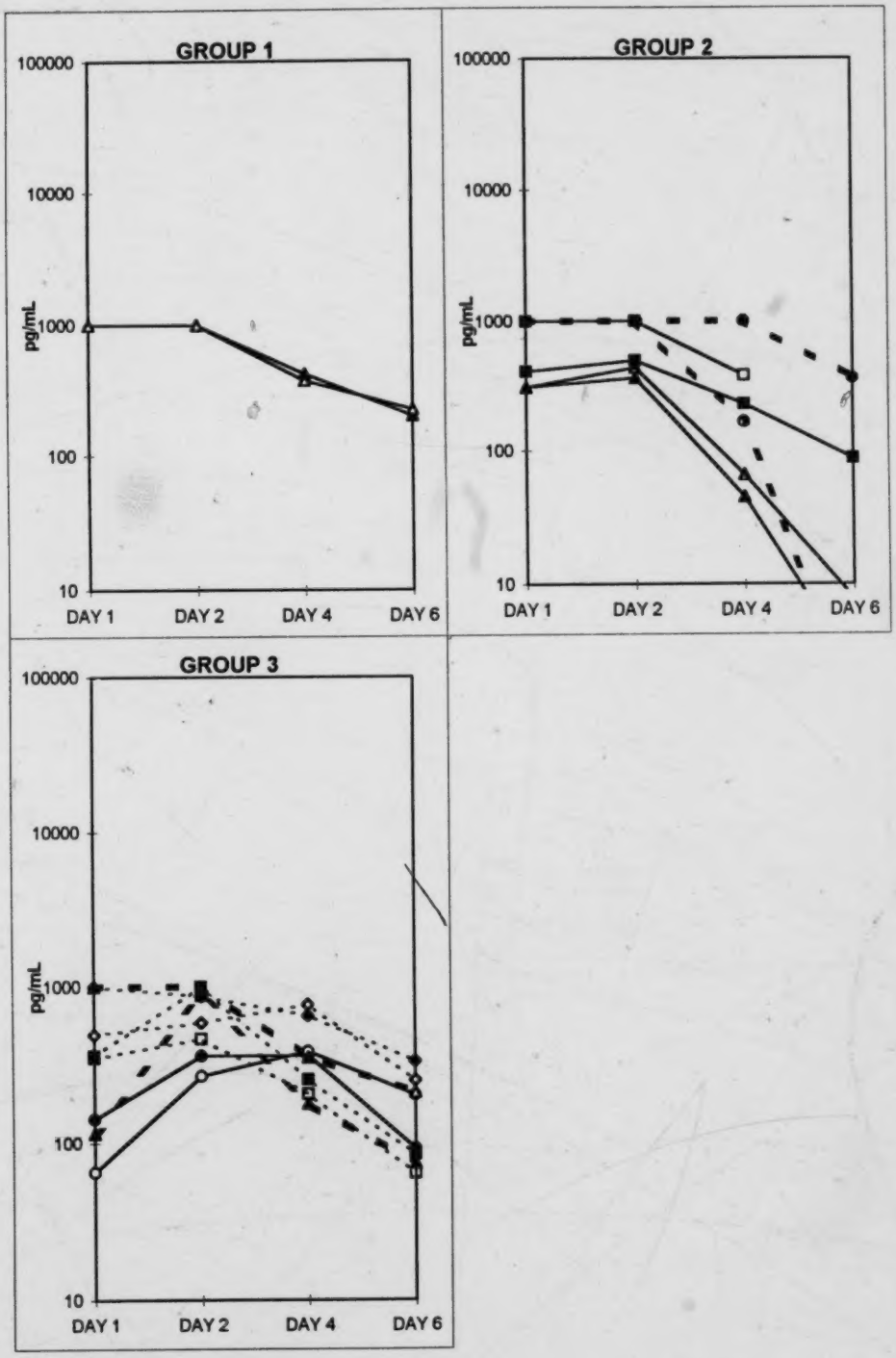


Figure A-20. TNFa production by PBMC upon stimulation with 2 u/mL of PHA.

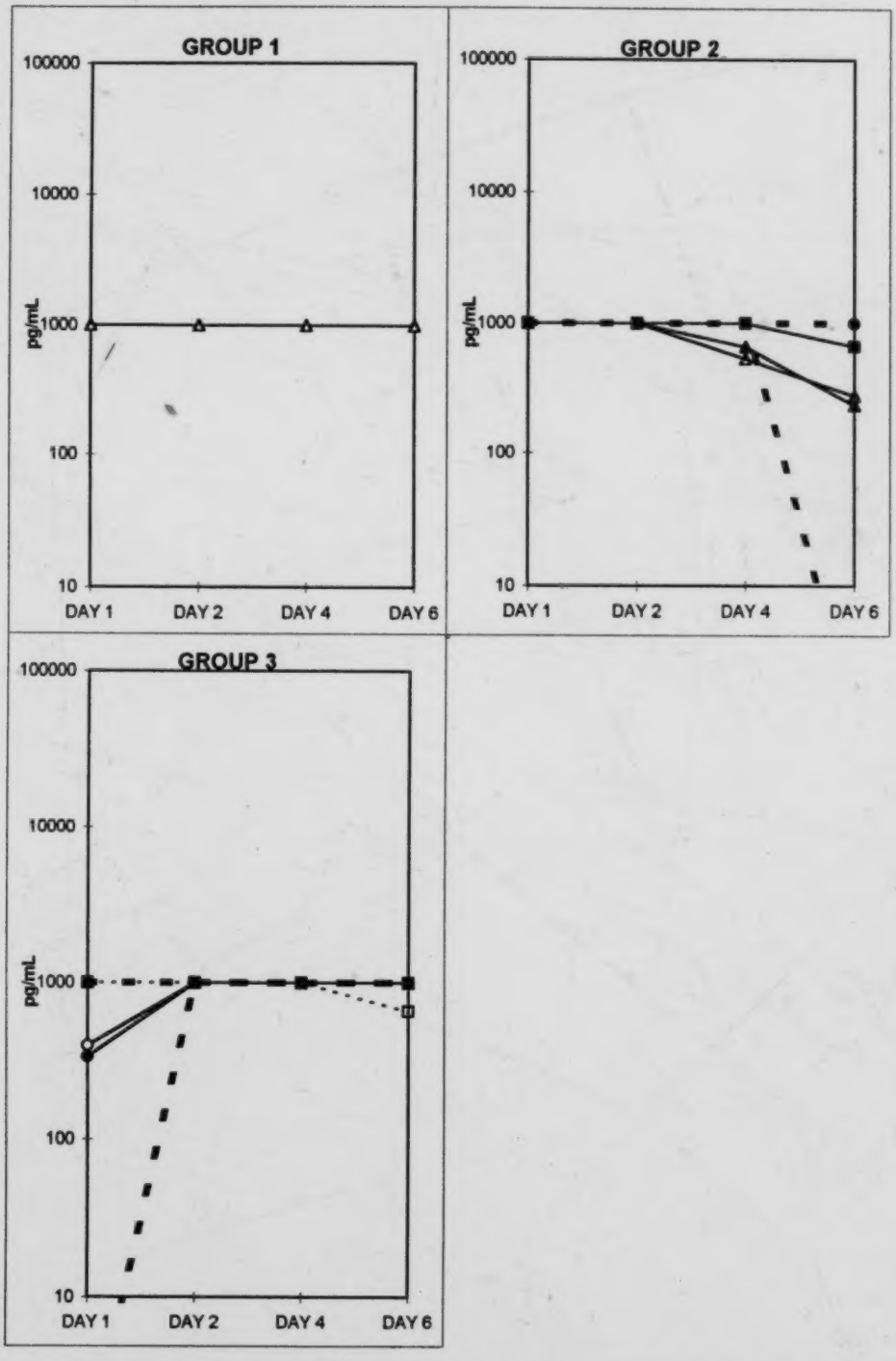


Figure A-21. TNFa production by PBMC upon stimulation with 10 ug/mL of PHA.

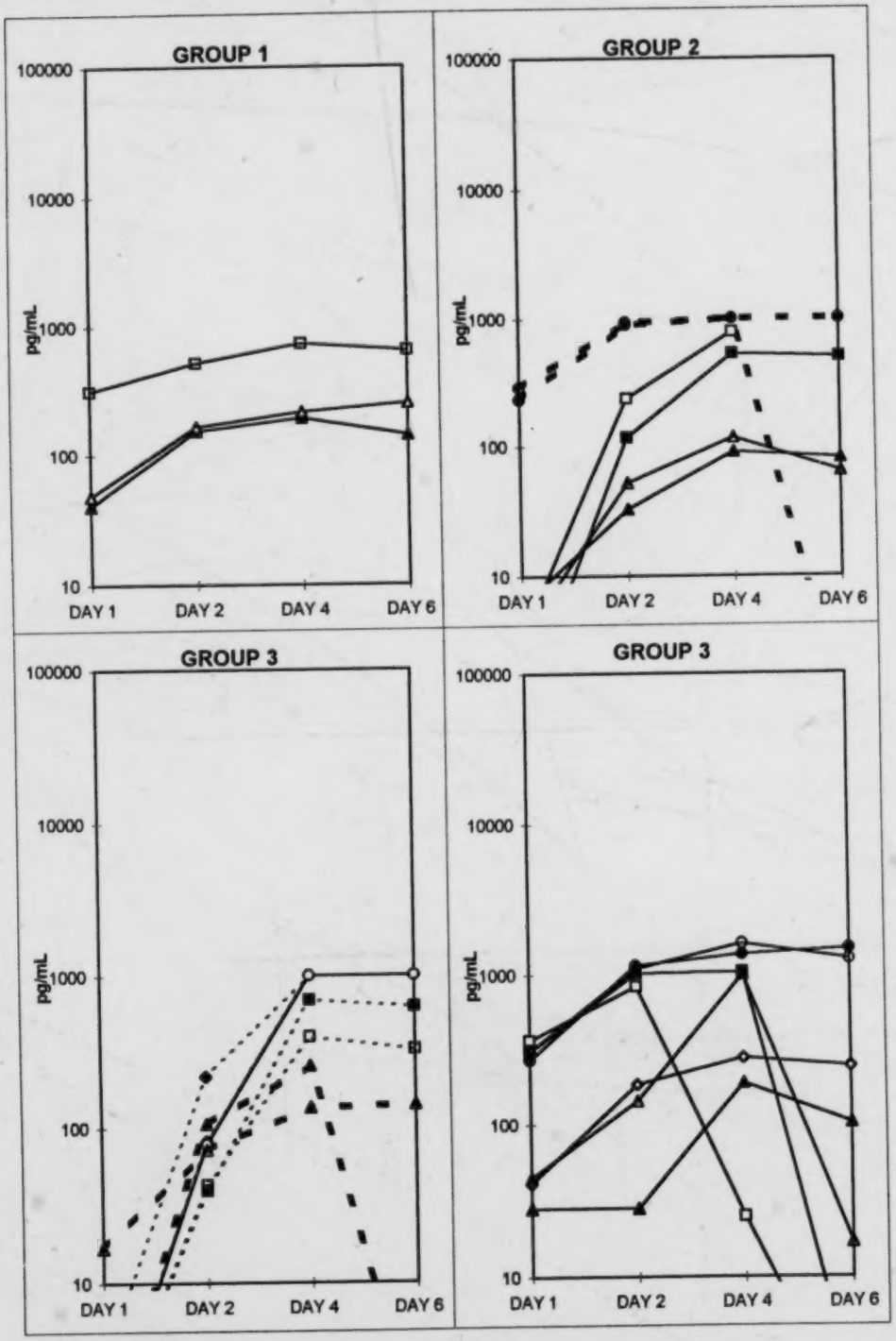
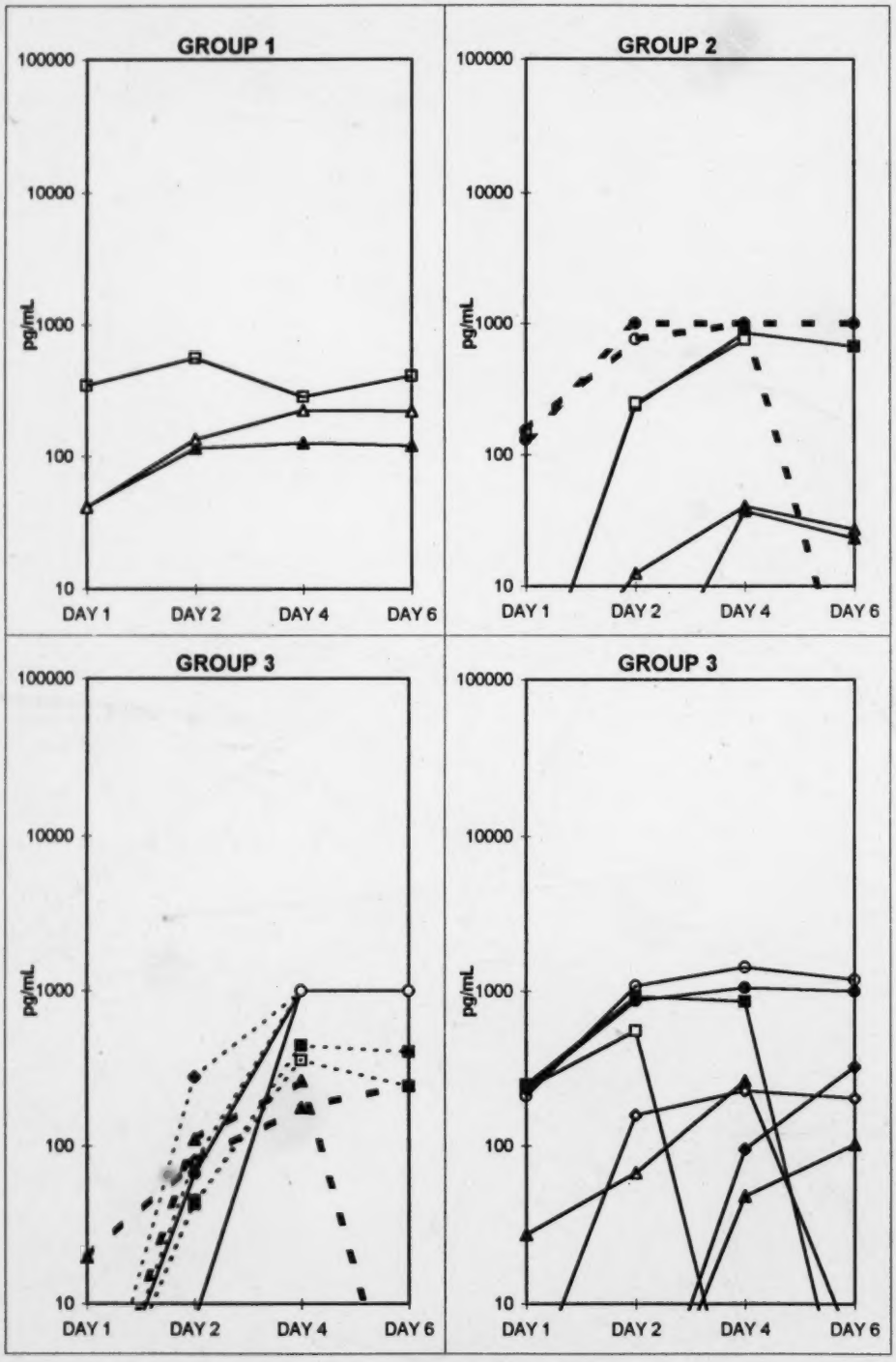


Figure A-22. IL5 production by PBMC upon stimulation with 10 ug/mL of PHA.



FigureA-23.L5 production by PBMC upon stimulation with 2 ug/mL of PHA.

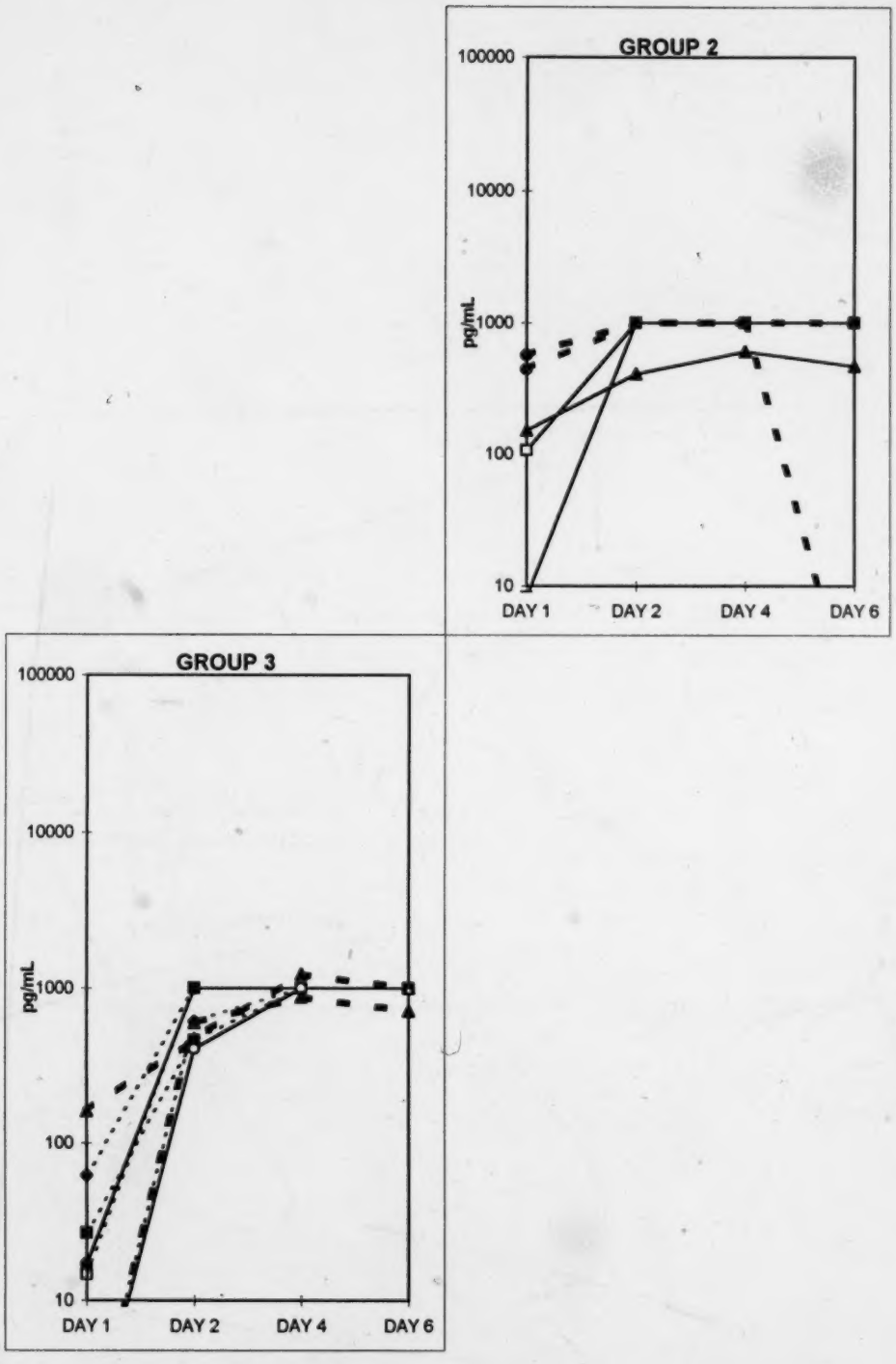
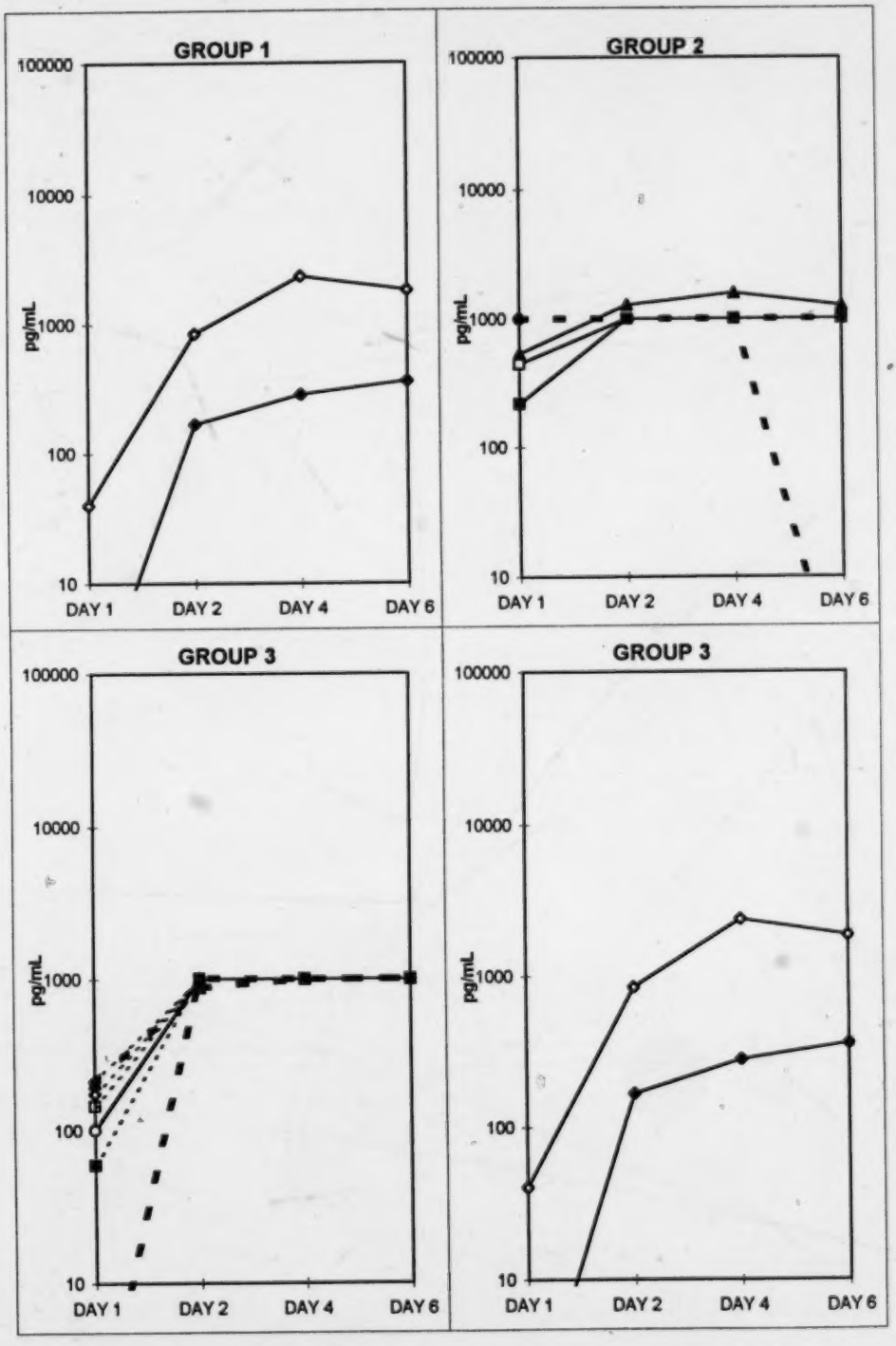


Figure A-24. IL13 production by PBMC upon stimulation with 2 ug/mL of PHA.



FigureA-25. IL13 production by PBMC upon stimulation with10 ug/mL of PHA.

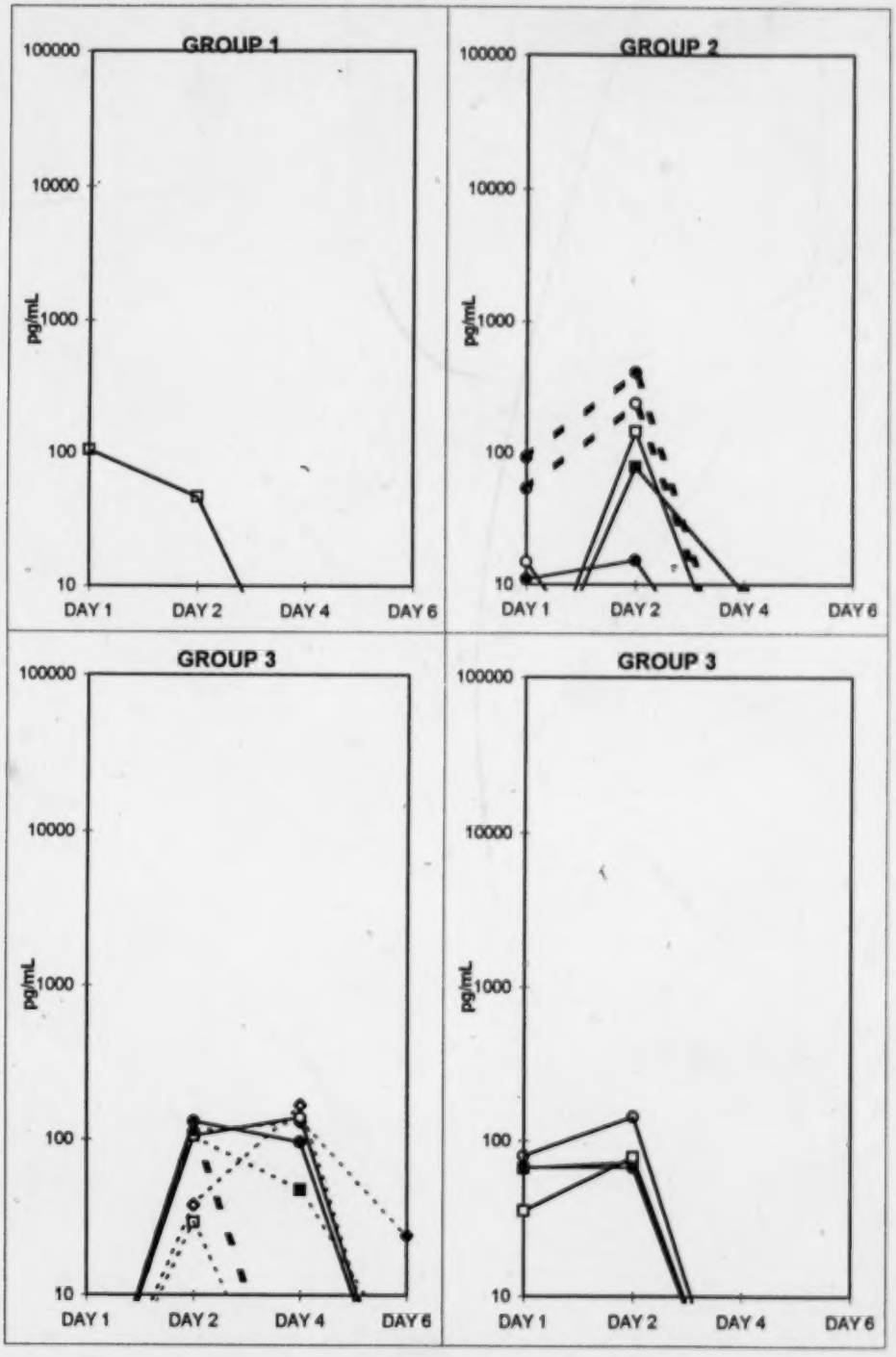


Figure A-26. IL4 production by PBMC upon stimulation with 2 ug/mL of PHA.

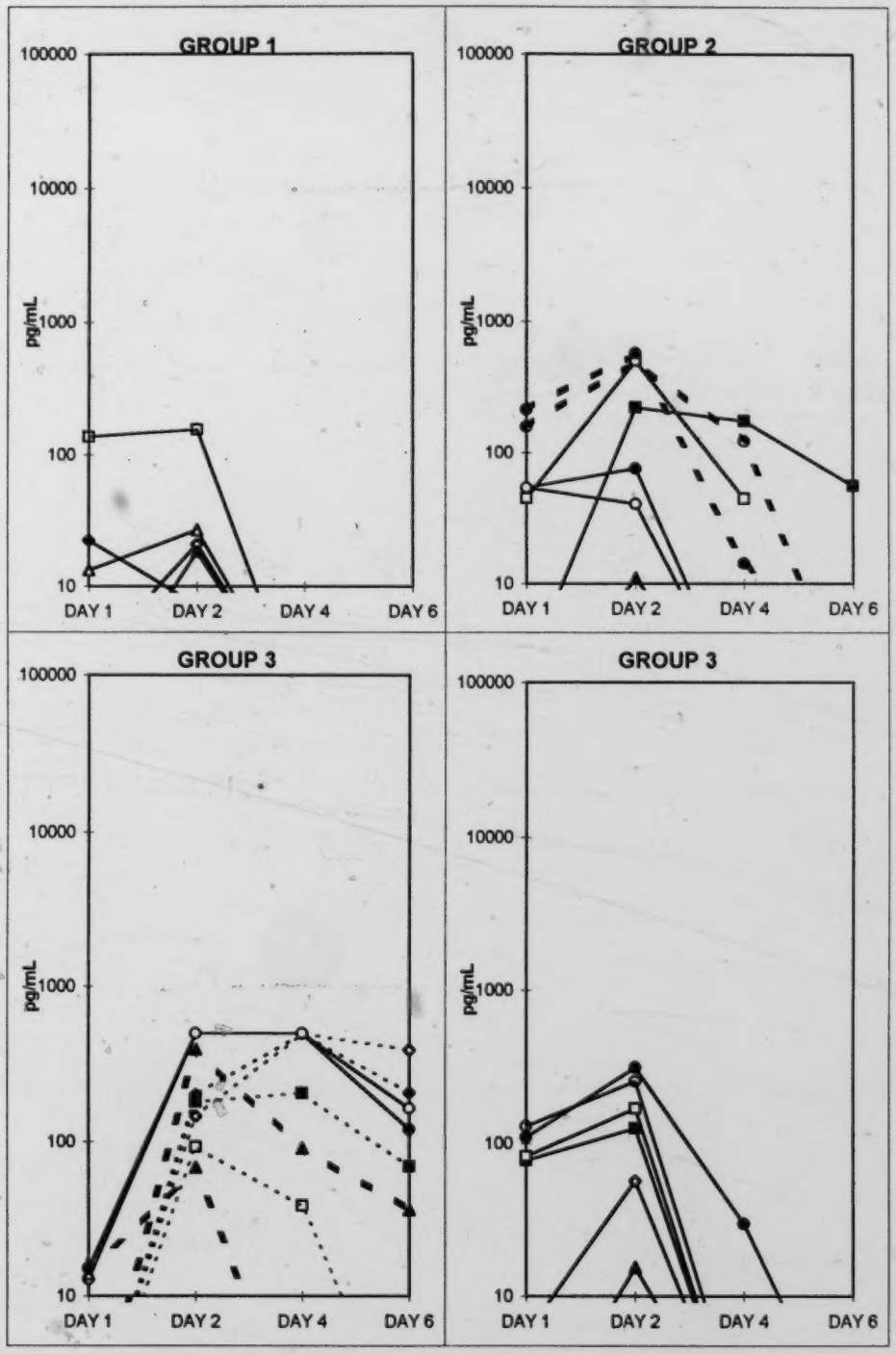
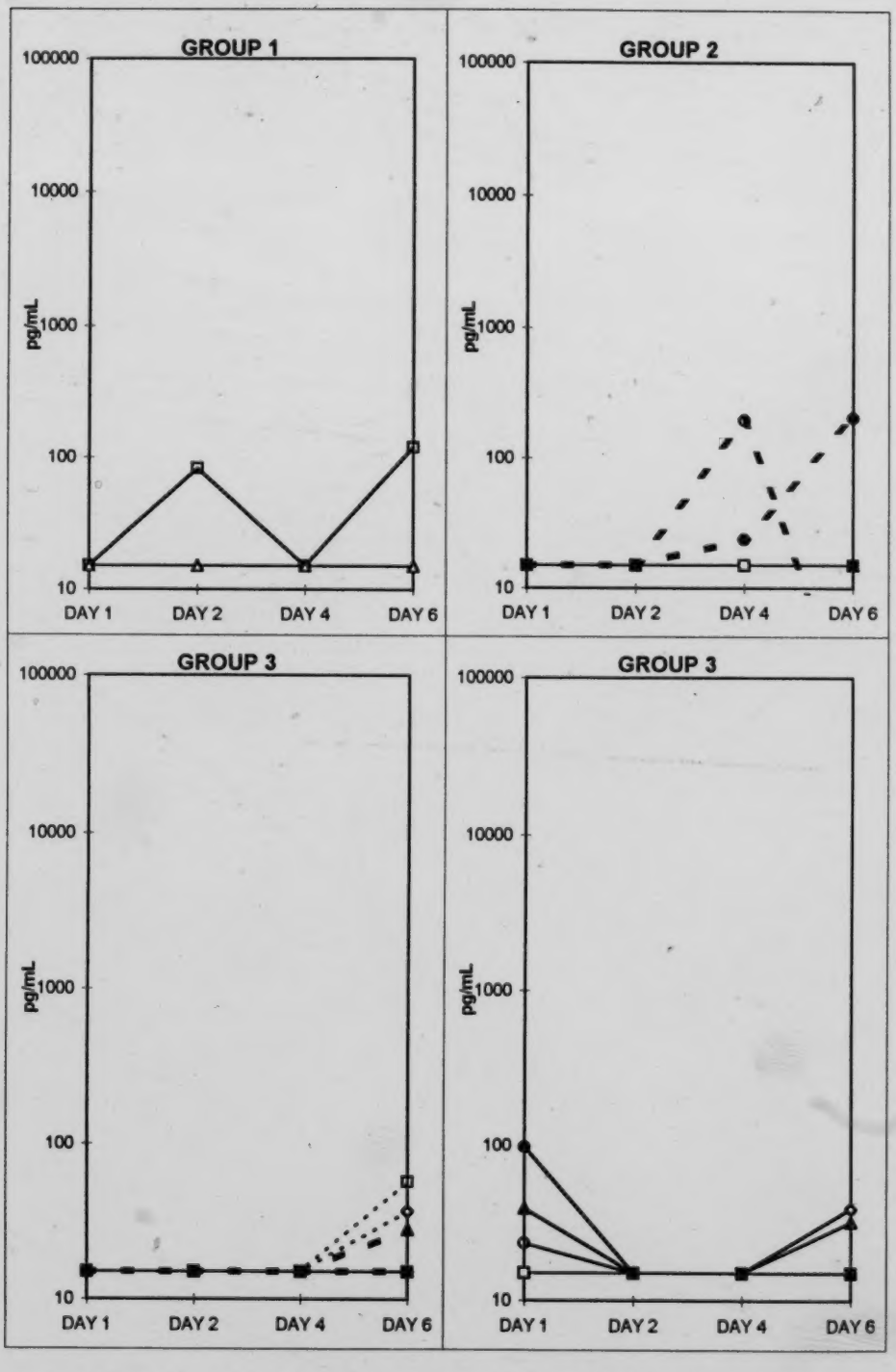


Figure A-27. IL4 production by PBMC upon stimulation with 10 ug/mL of PHA.



FigureA-28. IL5 production by PBMC without antigenic stimulation(control cultures).

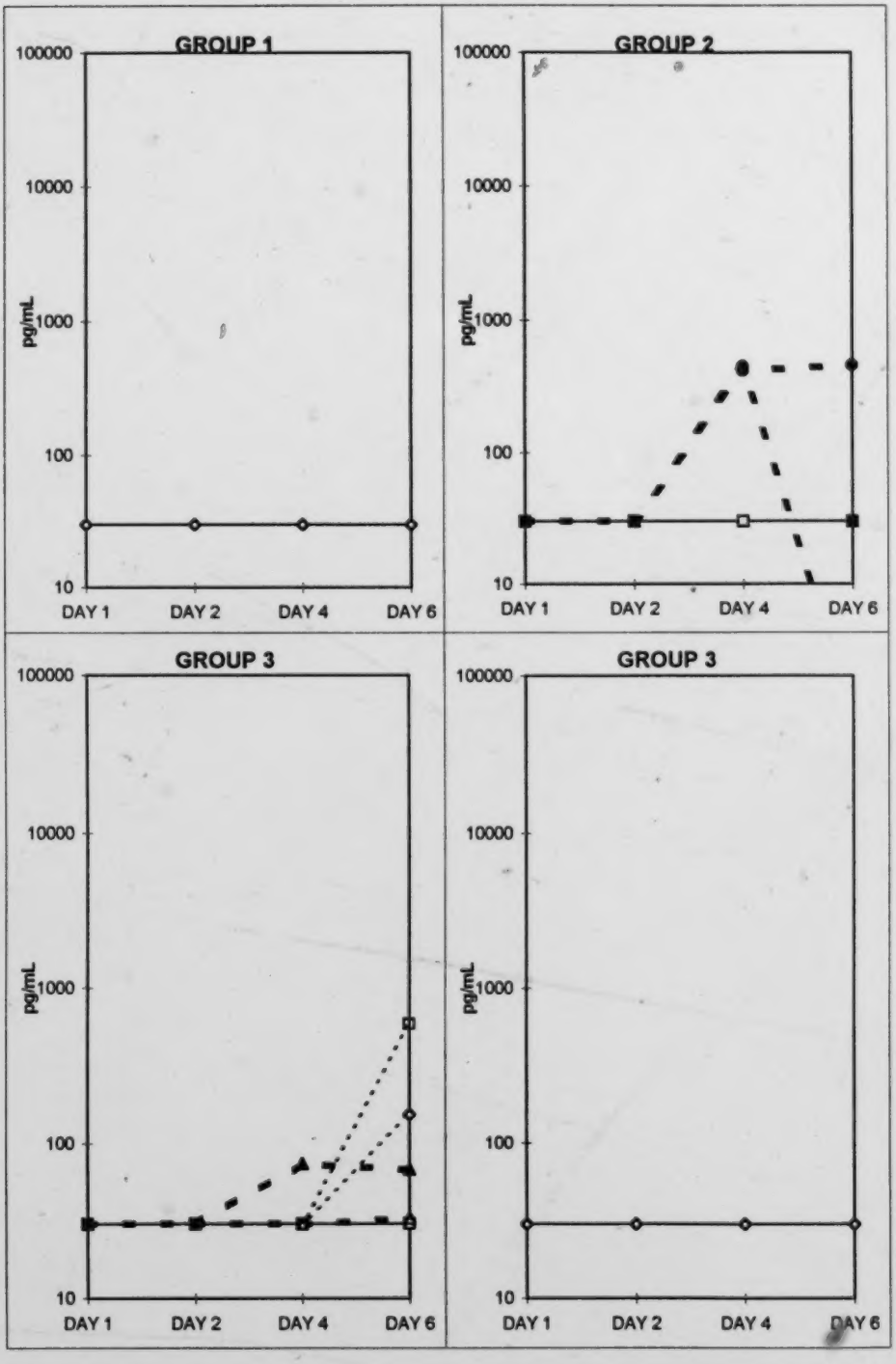


Figure A-29. IL13 production by PBMC without antigenic stimulation (control cultures).

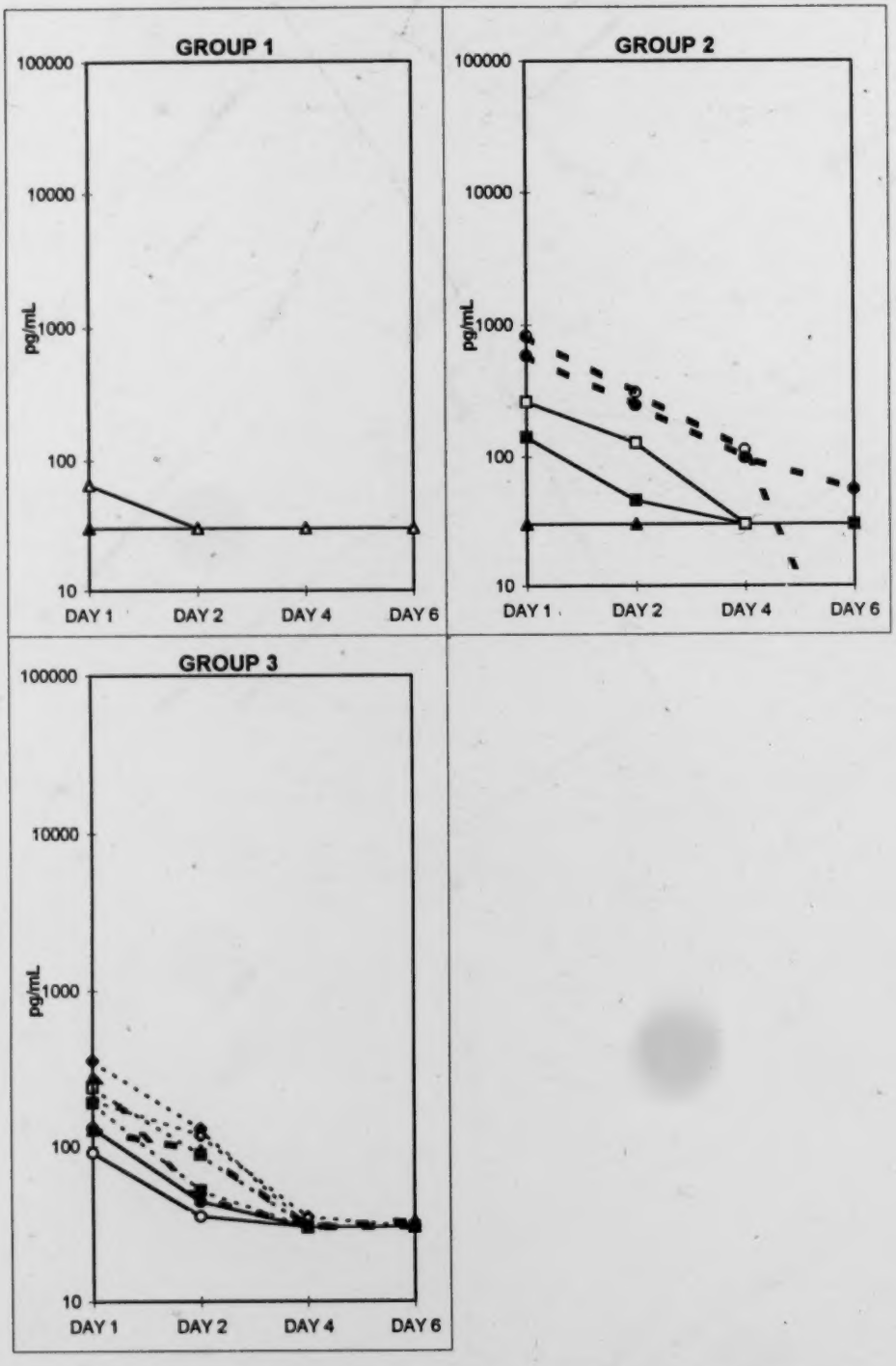


Figure A-30. TNFa production by PBMC without antigenic stimulation(control cultures).